Activation of adenosine A$_{2A}$ receptor by lipids from docosahexaenoic acid revealed by NMR

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The lipid composition of the plasma membrane is a key parameter in controlling signal transduction through G protein–coupled receptors (GPCRs). Adenosine A$_{2A}$ receptor (A$_{2A}$AR) is located in the lipid bilayers of cells, containing acyl chains derived from docosahexaenoic acid (DHA). For the NMR studies, we prepared A$_{2A}$AR in lipid bilayers of nanodiscs, containing DHA chains and other acyl chains. The DHA chains in nanodiscs enhanced the activation of G proteins by A$_{2A}$AR. Our NMR studies revealed that the DHA chains redistribute the multiple conformations of A$_{2A}$AR toward those preferable for G protein binding. In these conformations, the rotational angle of transmembrane helix 6 is similar to that in the A$_{2A}$AR–G protein complex, suggesting that the population shift of the equilibrium causes the enhanced activation of G protein by A$_{2A}$AR. These findings provide insights into the control of neurotransmissions by A$_{2A}$AR and the effects of lipids on various GPCR functions.

INTRODUCTION

G protein–coupled receptors (GPCRs) are one of the largest membrane protein families in eukaryotes. The GPCR family includes receptors of various neurotransmitters and hormones, and more than 30% of modern drugs target GPCRs. Drug binding to GPCRs leads to the induction or inhibition of signal transduction mediated by G proteins, β-arrestins, and various other effectors. Under physiological conditions, GPCRs are embedded in lipid bilayers, and accumulating evidence has shown that the signaling activities of GPCRs are affected by the surrounding lipids.

Adenosine A$_{2A}$ receptor (A$_{2A}$AR), a class A GPCR, controls inflammation, neurotransmission, blood flow, and immune responses (1). In the brain striatum, A$_{2A}$AR is involved in neurotransmissions that controls motor skills, and A$_{2A}$AR is a drug target for the reduction of dyskinesia in Parkinson’s disease (1).

In cells, A$_{2A}$AR is embedded in membranes containing acyl chains derived from docosahexaenoic acid (DHA), a polyunsaturated fatty acid with 22 carbons and six double bonds, and arachidonic acid (ARA), a polyunsaturated fatty acid with 20 carbons and four double bonds. Dietary DHA intake increases and decreases the populations of the DHA and ARA chains, respectively (2). In the lipid bilayers of the mammalian brain striatum, in which A$_{2A}$AR is highly expressed (1), DHA constitutes 12 to 14% of the phospholipid acyl chains, and the population of the DHA chains is remarkably decreased from 3 to 8% by feeding DHA-deficient diets (3, 4). The effects of DHA on the activities of A$_{2A}$AR are unknown, although the signaling activities of rhodopsin and cannabinoid CB1 receptor, which share ~10% sequence identity with A$_{2A}$AR, are reportedly affected by the population of the DHA chains in the lipid bilayer (5, 6). Clarification of the effects of DHA on the conformation and activity of A$_{2A}$AR would be helpful for understanding the signal transduction through A$_{2A}$AR under physiological conditions.

The three-dimensional structures of A$_{2A}$AR have been solved in various forms, including the inverse agonist bound, full agonist bound, and ternary complex composed of A$_{2A}$AR, a full agonist, and an engineered G protein, by x-ray crystallography and cryo–electron microscopy (7–10). In the structures of the ternary complex, the cytoplasmic half of transmembrane 6 (TM6) was shifted outward and rotated clockwise, when viewed from the cytoplasmic surface, to form a cavity in the cytoplasmic surface, as compared to the structures in the inverse agonist-bound form. The C-terminal helix of the engineered G protein was inserted into the cavity (7, 10). The cytoplasmic cavities were also found in the crystal structures of other GPCRs bound to G protein or arrestin (11). In the solution state, GPCRs exist in an equilibrium between inactive and active conformations, and the populations and exchange rates between these two conformations are related to their functions, as exemplified by nuclear magnetic resonance (NMR) and other spectroscopic studies (12). In the case of A$_{2A}$AR, the NMR signals originating from the isoleucine δ1-methyl groups (13) and chemically introduced CF$_3$ probes (14) indicated that the TM region of A$_{2A}$AR exchanges between multiple conformations in the presence of the full agonist. In addition, the resonances from the glycine NH and tryptophan Nε groups of A$_{2A}$AR were used to observe the allosteric modulation of W246 by the D52N mutation (15). However, little is known about the effects of the DHA chains on the conformations and the signaling activities of GPCRs. Therefore, the function-related dynamics of A$_{2A}$AR in lipid bilayers containing DHA chains must be clarified.

Reconstituted high-density lipoproteins (rHDLs), also known as nanodiscs (16), can accommodate membrane proteins within a 10-nm-diameter disc-shaped lipid bilayer. rHDLs reportedly provide lipid environments with more native-like properties, as compared with liposomes, in terms of the curvature and phase behavior (17). rHDLs and other disc-shaped lipid bilayers have been used in various in vitro GPCR studies (18–21), and our NMR studies of GPCRs in rHDL lipid bilayers revealed that the populations of the active conformation of GPCRs in rHDLs correlated better with the signaling levels than those in detergent micelles (20). These methods for the reconstruction of GPCRs into the lipid bilayers of rHDLs allow examination of the effects of the composition of lipid bilayers on the activities and conformational equilibria of GPCRs.

Here, we observed the NMR signals of the methionine residues of A$_{2A}$AR in the lipid bilayers of rHDLs, containing the DHA and
ARA chains, to identify the conformational equilibria related to the signaling activities. We found that the DHA chains in the rHDLs enhanced the G protein activation by A2A AR. On the basis of our observation of the conformational equilibria, we discuss the mechanism underlying the modulation of the signaling activity of A2A AR by the DHA chains.

RESULTS
Preparation and characterization of A2A AR in rHDL
Pichia pastoris was used as the host for the large-scale expression of functional A2A AR. A2A AR was solubilized in n-dodecyl-β-d-maltopyranoside (DDM) and purified by two purification steps. For the investigation of the conformational dynamics of A2A AR in lipid bilayers, purified A2A AR in DDM micelles was reconstituted into rHDLs with a mixture of 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleyl-phosphatidylglycerol (POPG). The population of the anionic lipid (POPG) was 25% (POPC:POPG = 3:1), which is similar to the population of the anionic lipids in mammalian cell membranes (22). Here, the rHDLs prepared from the POPC and POPG lipids are referred to as rHDL(POPC/POPG). The size exclusion chromatography revealed that purified A2A AR in rHDL(POPC/POPG) is monodisperse, with a Stokes diameter of 12 nm (fig. S1A), in good agreement with the previously reported rHDL size. As judged from SDS–polyacrylamide gel electrophoresis analyses, the purity of A2A AR in rHDL(POPC/POPG) was >90%, and the ratio of A2A AR to MSP1E3 was consistent with the 1:2 stoichiometry that would be expected when each rHDL particle is composed of two MSP1E3 molecules and one A2A AR (fig. S1B).

Radioligand-binding assays with an excess amount of a tritium-labeled NECA of [N-ethylcarboxamido]adenosine (NECA), which has a higher affinity for A2A AR than the endogenous ligand adenosine, revealed that these mutants exhibited the ligand binding activities after a 24-hour incubation at 298 K (fig. S3G). In the 1H-13C HMQC spectra of [(α,β,β,-2H, methyl-13C]-Met, (α,β,-2H]-A2A AR by the DHA chains.

NMR resonances from methionine residues of A2A AR in rHDLs
To observe the NMR resonances from A2A AR in rHDLs with a molecular mass of ~180 kDa, composed of ~35 kDa A2A AR, ~80 kDa lipids, and ~60 kDa MSP1E3, we used 13C selective labeling of the methionine methyl groups, which reflect the spatial arrangement of the neighboring aromatic residues (12), along with sensitivity enhancement by deuterium. The deuteration and methionine methyl–selective 13C labeling in the yeast expression system were accomplished by using medium containing deuterium oxide and [α,β,β,-2H, methyl-13C] methionine. In the 1H-13C heteronuclear multiple-quantum coherence (HMQC) spectrum of [(α,β,β,-2H, methyl-13C]-Met, (α,β,-2H]-A2A AR in DDM micelles bound to the inverse agonist, resonances that apparently originate from six methionine residues were observed (fig. S2A). For the assignment of the methionine resonances, we mutated the methionine residues. For example, M211(5,72) superscripts indicate GPCR database (GPCRdb) numbering (25) was assigned by introducing the M211T mutation into A2A AR. A resonance was absent in the spectrum of the M211T mutant, revealing that this resonance is from M211(5,72) (fig. S2B). The resonances from the other methionine residues were assigned in a similar manner (fig. S2, C to G). Assignments for the methionine residues in the presence of the full agonist were accomplished in the same manner as those in the presence of the inverse agonist (fig. S2, H to L). The resonances from A2A AR in rHDLs were assigned by comparison with the spectra of A2A AR in micelles. In the spectra of [(α,β,β,-2H, methyl-13C]-Met, (α,β,-2H]-A2A AR in rHDL(POPC/POPG) bound to the inverse agonist, the resonances with chemical shifts similar to those of M140(4,6), M174(4,6), and M211(5,72) in DDM micelles were observed, revealing that they are from M140(4,6), M174(3,7), and M211(5,72) (fig. S2M). The resonances corresponding to M140(4,6) and M211(5,72) of A2A AR in rHDL(POPC/POPG) bound to the full agonist were assigned in a similar manner (fig. S2N).

To investigate the conformation of A2A AR at the cytoplasmic surface, we newly introduced methionine residues as structural probes to the cytoplasmic surface by the I106M, A232M, V239M, and I292M mutations (Fig. 1B), according to the comparison with other class A GPCRs: The residues corresponding to I106(3,54), A232(3,54), V239(4,6), and I292(4,7) are methionine in at least one of the class A GPCRs. We confirmed that these mutants retained the ligand binding activity (fig. S3, A to E) and the G protein signaling activity (fig. S3F) and that these mutants exhibited the ligand binding activities after a 24-hour incubation at 298 K (fig. S3G). In the 1H-13C HMQC spectra of [(α,β,β,-2H, methyl-13C]-Met, (α,β,-2H]-A2A AR in rHDL(POPC/POPG) with the A232M, V239M, I292M, or I106M/I292M mutations, bound to the inverse agonist (fig. S4, A to D) and the full agonist (fig. S4, E to H), one resonance was additionally observed upon the methionine residue introduction, revealing that these resonances are from M232(6,34), M239(6,4), M292(6,47), and M106(3,54). The remaining methionine methyl signals did not exhibit substantial chemical shift changes in the presence of these mutations, and thus, they did not affect the global conformation of A2A AR. The chemical shifts of the resonances from M106(3,54), M140(4,6), M211(5,72), M232(3,54), M239(6,34), and M292(6,47) of A2A AR in rHDL(POPC/POPG) in the presence of the inverse agonist were markedly different from those in the presence of the full agonist (Fig. 1, C to G), suggesting that a large conformational change occurs upon A2A AR activation.

Conformational equilibrium of A2A AR in rHDLs bound to the full agonist and partial agonist
To examine the resonances undergoing conformational exchanges, we also recorded the spectra at lower and higher temperatures, 288 and 308 K, respectively. In the spectra observed at 288 and 308 K, the resonance from M106(3,54) remarkably shifted from that at 298 K (fig. S5, A and B). In addition to the resonance from M106(3,54), the resonances from M140(4,6) and M292(6,47) exhibited the temperature-dependent shift (fig. S5, C to F). In the cases of M140(6,4) and M292(6,47), the chemical shift changes corresponding to these temperature-dependent shifts were not induced by the type of surrounding lipids (vide infra). Thus, these temperature-dependent shifts reflect other conformational equilibria, which are not regulated by the lipids. The M211(5,72) signals exhibited almost identical chemical shifts at 288,
Fig. 1. Conformations of A2A AR in rHDLs bound to the inverse agonist, full agonist, and partial agonist. (A) [35S]-GTPγS binding to complexes of the purified G protein, A2A AR in rHDL(POPC/POPG), and the ligands. Results are expressed as the initial rates of the production of the GTPγS and G protein complex. Data are the means ± SEM of triplicate determinations from three separate representative experiments. (B) Distribution of the residues with NMR signals observed for A2A AR in rHDLs in the crystal structure of A2A AR. The crystal structure of A2A AR with a full agonist, NECA [Protein Data Bank (PDB) code: 2YDV], is shown in ribbons in a side view with the extracellular sides on the top. I106(M) 3.54, M140 4.61, M211 5.72, A232(M) 6.34, V239(M) 6.41, and I292(M) 8.47 are depicted by black sticks. M174 5.37, M177 5.40, M193 5.54, and M270 7.34 are depicted by gray sticks. NECA is depicted by gray sticks. The membrane position generated by the Orientations of Proteins in Membranes (OPM) database (http://opm.phar.umich.edu/) is indicated. (C) Schematic diagrams of A2A AR in rHDL(POPC/POPG) bound to the inverse agonist and full agonist. (D to G) Overlaid 1H-13C HMQC spectra of A2A AR (D), A2A AR/A232M (E), A2A AR/V239M (F), and A2A AR/I106M/I292M (G), labeled with [(α,β,β-2H, methyl-13C] Met, u-2H], bound to the inverse agonist (blue) and the full agonist (orange). Only the regions with methionine methyl resonances are shown. The M1404.61 and M2115.72 signals in (D), the M2326.34 signals in (E), the M2396.41 signals in (F), and the M1063.54 and M2928.47 signals in (G) are indicated and connected with lines. (H) 1H-13C HMQC spectra of [(α,β,β-2H, methyl-13C] Met, u-2H] A2A AR/I106M/I292M in rHDL(POPC/POPG) in the presence of the partial agonist. Cross sections at the dashed lines are shown above the spectrum. Superscripts I and A are added to the labels of the M1063.54, M1404.61, M2115.72, and M2928.47 signals with chemical shifts similar to those in the presence of the inverse agonist and the full agonist, respectively. The M1404.61 signal, which is lower than that of the minimum contour level, is enclosed in boxes and shown at the left of the spectrum with different contour levels. In (D) to (H), only the regions with methionine methyl resonances are shown, and the centers of the methionine resonances are indicated with dots.
DHA chains enhanced G protein activation by A2AAR

To investigate the effects of the DHA chains on the conformation and signalling activities of A2AAR, A2AAR was reconstituted into rHDLs composed of lipids with the DHA or ARA chains. Here, rHDLs containing the DHA and ARA chains are referred to as rHDL(DHA) and rHDL(ARA), respectively. Lipids containing 1-stearoyl-2-docosahexaenoyl-phosphocholine (SDPC), 1-stearoyl-2-docosahexaenoyl-phosphoglycerol (SDPG), and 1-stearoyl-2-arachidonoyl-phosphocholine (SAPC), in which one of the acyl chains is derived from DHA or ARA, were used for the preparation of A2AAR in rHDL(DHA) and A2AAR in rHDL(ARA) (fig. S6A). Considering the fact that more than 40% of the phospholipid acyl chains in cellular membranes are composed of those derived from DHA or ARA (26), we prepared A2AAR in rHDL(DHA) and A2AAR in rHDL(ARA) using lipids with SDPC:POPG:POPC:POPG = 9:3:3:1 and SAPC:POPG = 3:1, respectively, in which 40% of the total acyl chains are derived from DHA or ARA (fig. S6A). The populations of the anionic lipids (SDPG, SAPG, and POPG) of A2AAR in rHDL(DHA) and A2AAR in rHDL(ARA) were the same as those of A2AAR in rHDL(POPC/POPG) (fig. S6A).

We used NMR to examine the lipid composition of A2AAR in rHDLs. In the 1H-1D spectrum of A2AAR in rHDL(DHA), an ensemble of resonances from the methyl groups of the DHA chains and another ensemble of resonances from the methyl groups of the other acyl chains were observed with 4:6 signal intensity ratio, suggesting that DHA constitutes 40% of the acyl chains in A2AAR in rHDL(DHA) (fig. S6B). In the 1H-1D spectrum of A2AAR in rHDL(ARA), a resonance from the methyl groups of the polar head of SAPC, an ensemble of resonances from methylene groups connecting the C=C double bonds in the ARA chain, the methylene groups farther away from the C=C double bonds, and the methylene groups of the acyl chains were observed (fig. S6C). The signal intensities of these resonances indicate that ARA constitutes ~35% of the fatty acids in A2AAR in rHDL(ARA). Therefore, the populations of DHA and ARA in the acyl chains are almost identical to those of the lipids used for the preparation of the rHDLs. In the 31P-1D spectrum of A2AAR in rHDL(POPC/POPG) and A2AAR in rHDL(DHA), the resonances from phosphatidylcholine and phosphoatidylglycerol were observed with a 3:1 signal intensity ratio (fig. S6D), suggesting that the population of the polar head groups is comparable to those of the lipids used for the preparation of the rHDLs. In the tritium-labeled full agonist binding assays, A2AAR in rHDL(DHA) and A2AAR in rHDL(ARA) exhibited almost identical dissociation constants (150 and 140 nM, respectively; fig. S6, E and F).

We examined the time course of the [35S]-GTPγS binding to the complex of A2AAR in rHDL(DHA), with the heterotrimeric G protein and the full agonist. As a result, the initial [35S]-GTPγS binding rate for A2AAR in rHDL(DHA) was 1.88 ± 0.07 times higher than that for A2AAR in rHDL(POPC/POPG) (fig. 2B). In contrast, the initial [35S]-GTPγS binding rate for A2AAR in rHDL(ARA) was only 1.10 ± 0.07 times higher than that for A2AAR in rHDL(POPC/POPG) (fig. 2B).

There are differences between rHDL(POPC/POPG), rHDL(ARA), and rHDL(DHA) in the hydrocarbon chain lengths of the sn-1 chains. To examine the effects of the differences between samples in terms of the hydrocarbon chain length of the sn-1 chains, we compared the rHDLs with identical lipid compositions, except for the substitution of the DHA chains with other acyl chains. We examined the time course of the [35S]-GTPγS binding to A2AAR in rHDLs using lipids with SDPC:POPG = 3:1, in which the lipid composition is identical to rHDL(ARA) except for the substitution of the DHA chains for the palmitoyl and oleic acid chains (fig. S7C), exhibited a higher initial [35S]-GTPγS binding rate than A2AAR in rHDL(POPC/POPG) (fig. S7D).
Effect of lipids on the conformational equilibrium of A2A AR in rHDLs

To investigate the effects of the lipids on the conformational equilibrium of A2A AR in rHDLs, the 1H-13C HMQC spectra of A2A AR/1106M/I292M, labeled with [α,β,β,2-H, methyl-13C] Met, u-2H] and embedded in rHDL(DHA) and rHDL(ARA), were observed in the presence of the partial agonist. In these spectra, M1066.54, M1407.34, M2117.52, and M2928.64 exhibited two resonances, and their chemical shifts were similar to those of the inactive (M106, M140, M211, and M292) and (Fig. 3, A to C). The intensity ratios of the active state signals, relative to the inactive ones, of A2A AR in rHDL(DHA) were less than 1.1-fold higher than those of rHDL(ARA), suggesting that the populations of the inactive and active states are not affected by the type of surrounding lipids, which cannot explain the 1.7-fold higher GDP/GTP reaction rate of A2A AR in rHDL(DHA) than A2A AR in rHDL(ARA) (Fig. 2B). In contrast, the M106 signal of A2A AR in rHDL(DHA) exhibited a remarkably different chemical shift from that of A2A AR in rHDL(DHA) (Fig. 3, D and E). Comparisons of the M106 signals of A2A AR in rHDL(DHA), rHDL(ARA), and rHDL(POPC/POPG) revealed that the M106 signal of A2A AR in rHDL(DHA) was observed at a chemical shift in between those of A2A AR in rHDL(DHA) and A2A AR in rHDL(POPC/POPG) (Fig. 3, D to G), suggesting that, in the presence of the partial agonist, the amount of each substrate in the active state of A2A AR is affected by the type of surrounding lipids.

To further investigate the effects of lipids on the equilibria among the multiple conformations in the active state of A2A AR, 1H-13C HMQC spectra of A2A AR/1106M/I292M, A2A AR/A232M, and A2A AR/V239M, labeled with [α,β,β,2-H, methyl-13C] Met, u-2H] and embedded in rHDL(DHA) or rHDL(ARA), were observed in the presence of the full agonist (Fig. 4A). In the spectra, the resonances from M1066.54 and M2326.34 of A2A AR in rHDL(ARA) were markedly different from those of A2A AR in rHDL(DHA), and the chemical shifts of the M1066.54 and M2326.34 signals of A2A AR in rHDL(ARA) were in between those of A2A AR in rHDL(DHA) and A2A AR in rHDL(POPC/POPG) (Fig. 4, B and C). These residues exist at the cytoplasmic ends of TM3 and TM6 (Fig. 4E) and are >8 Å away from the lipid bilayers and thus would be less affected by direct interactions with lipids. The chemical shifts of the M1406.61, M2117.52, M2397.41, and M2928.64 signals, which are on the flexible loop region, were examined by solvent paramagnetic relaxation enhancement (PRE) experiments. In these experiments, Gd-diethylentriamine pentaaetic acid-bis[methylamide] (Gd-DTPA-BMA), a highly water-soluble paramagnetic probe that lacks a site for protein binding (27), was added to the sample containing A2A AR in rHDL (Fig. 5A). The PRE arising from the magnetic dipolar interactions between the nuclear spin and the unpaired electron spin of the diffusing paramagnetic probe enhances the relaxation of the nuclear spins in a distance-dependent manner, leading to the intensity reduction of the NMR signals of the solvent-exposed residues (Fig. 5A). In the 1H,13C HMQC spectrum of A2A AR in rHDL(DHA) in the presence of the full agonist and Gd-DTPA-BMA, the M2326.34 signal intensity was remarkably lower than that in the absence of Gd-DTPA-BMA (Fig. 5B). In the case of A2A AR in rHDL(DHA), the M2326.34 signal intensity was more affected by Gd-DTPA-BMA than that of A2A AR in rHDL(ARA) (Fig. 5C), suggesting that M2326.34 is more accessible to the solvent in rHDL(DHA) than in rHDL(ARA). In contrast, the M1066.54 signal intensity of A2A AR in rHDL(DHA) was less affected by Gd-DTPA-BMA than that of A2A AR in rHDL(ARA) (Fig. 5, D and E), suggesting that M1066.54 has lower solvent accessibility in rHDL(DHA) than in rHDL(ARA). The Gd-DTPA-BMA–induced intensity reductions observed for the M1406.61, M2117.52, and M2928.64 signals of A2A AR in rHDL(DHA) were almost identical to those of A2A AR in rHDL(ARA) (Fig. 5, F to M).

DISCUSSION

Effects of the DHA chains on the function-related dynamics of A2A AR

Our NMR experiments with A2A AR in rHDLs revealed that A2A AR, in the presence of the partial agonist, exists in equilibrium between the active state, which includes multiple substates, and the inactive state (Fig. S5, G to I) and that the full agonist skews the populations of these states toward the active state (Fig. S5, A, B, and F). In addition, the DHA chains induced the distribution change in the multiple active substates of A2A AR bound to the partial agonist and full agonist (Figs. 3 and 4). These effects of lipids on the conformational equilibrium would not be observed in A2A AR experiments under conditions using micelles, thus highlighting the importance of the utilization of rHDLs in this study.

On the basis of the comparison between the A2A AR crystal structures in the forms bound with an inverse agonist and a G protein mimetic, the following structural mechanism for the A2A AR activation was proposed (fig. S8) (7–9). The full agonist directly induces the movements of H2506.52, T883.36, W2466.48, S2777.42, and H2787.43, which, in turn, induce the repositioning of V1865.47, P1895.50, I923.40, F2426.44, and N2807.45 along with the movements of the intracellular sides of TM3, TM5, TM6, and TM7 (fig. S8). The movement of TM6 requires the reorganization of the side chains of Y1975.58 and Y2887.53. Y2887.53 constitutes a highly conserved NPxxY motif with N2847.49 and P2857.50, and the side-chain rotamer of Y2887.53 determines the activation state of the NPxxY motif (fig. S8) (28). The reorganization of the Y2887.53 side chain allows R1022.50 to adopt a fully extended conformation, packing against the side chain of Y391 in the α5 helix of the engineered G protein (fig. S8). Therefore, the side-chain rotamer of Y2887.53 is sensitive to the conformational change upon A2A AR activation. The 1H chemical shift of the M2928.47 methyl signals depends on the ring current effects from the neighboring aromatic ring of Y2887.53. The 1H chemical shifts of M2928.47 in the presence of
Fig. 3. Conformations of A2A AR in rHDLs with various lipid compositions bound to the partial agonist. (A to C) $^1$H-$^{13}$C HMQC spectra of [($\alpha$-,$\beta$,-$^2$H, methyl-$^{13}$C] Met, u-$^2$H] A2AAR/I106M/I292M in rHDL(DHA) (A), in rHDL(ARA) (B), and in rHDL(POPC/POPG) (C) bound to the partial agonist. Cross-sections at the dashed lines are shown above each spectrum. The methionine methyl resonances are indicated, and the centers are indicated with dots. Superscripts I and A are added to the labels of the M106$^{3.54}$, M140$^{4.61}$, M211$^{5.72}$, and M292$^{8.47}$ signals with chemical shifts similar to those in the presence of the inverse agonist and the full agonist, respectively. The M140 signals, which are lower than the minimum contour level, are enclosed in boxes and shown at the left of each spectrum with different contour levels. The methionine resonances are indicated, and the centers are indicated with dots. The spectra in (A), (B), and (C) are overlaid in (D) and (E). Only the region with M106 signals is shown in (E). The spectrum in Fig. 3C is identical to that in Fig. 1H, except for the color. (F) Normalized chemical shift differences of the methionine methyl resonances between A2A AR in rHDL(POPC/POPG) and A2A AR in rHDL(DHA), $\Delta \delta$, calculated by the equation $\Delta \delta = (\Delta \delta_{1H})^2 + (\Delta \delta_{13C}/3.5)^2$ as reported previously (36). Error bars represent SDs of the chemical shifts of 100 synthetic time domain data from the in situ error analysis (40). (G) Distribution of the methionine residues in the crystal structure of A2A AR. The crystal structure of A2A AR with a full agonist, NECA (PDB code: 2YDV), is shown in ribbons in a side view, with the extracellular sides on the top. I106(M)$^{3.54}$, which exhibited different chemical shifts between A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA), is depicted by purple sticks. M140$^{4.61}$, M211$^{5.72}$, and I292(M)$^{8.47}$, in which the chemical shifts of A2A AR in rHDL(DHA) were almost identical to those of A2A AR in rHDL(ARA), are depicted by dark gray sticks. M174$^{5.37}$, M177$^{5.40}$, M193$^{5.54}$, and M270$^{7.34}$, in which the resonances were not observed in the presence of the full agonist, are depicted by gray sticks. NECA is depicted by gray sticks. The membrane position generated by the OPM database (http://opm.phar.umich.edu/) is indicated.
of the inverse agonist [2.23 parts per million (ppm)] exhibited a larger downfield ring current shift than that in the presence of the full agonist (1.9 ppm) (Fig. 1G and fig. S4, C and G). In the crystal structures of A2A AR in the inverse agonist-bound form, the ring current from Y288 7.53 should induce a downfield shift in the 1H methyl atoms of M292 8.47, whereas Y288 7.53 is further away from M292 8.47 in the crystal structure of the ternary complex form with a full agonist and a G protein mimetic (fig. S8). These structural characteristics are in good agreement with the above-described conformations indicated by the 1H chemical shifts, suggesting that the inactive and active states correspond well to the states containing the inactive and active NPxxY motifs: NPxxYoff and NPxxYon, respectively.

The relative intensity of the M211A signal exhibited a strong correlation with the G protein activation rates observed for A2A AR in the presence of the inverse agonist, partial agonist, and full agonist (Fig. 6A), suggesting that the population of the NPxxYon conformations in the equilibrium is closely related to the levels of the G protein signaling activities. Various class A GPCRs also exist in equilibria between the inactive and active states, and there are good correlations between the population of the active state and the ligand efficacies (12). We found that the DHA chains enhanced the G protein activation...
Fig. 5. Conformations of A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA) examined by solvent PRE experiments. (A) Schematic diagrams of the solvent PRE experiments. (B to K) Overlaid cross sections of the resonances from M232^6.34, M106^3.54, M140^4.61, M211^5.72, and M292^8.47 in the $^1$H-$^1$H HMQC spectra of A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA), without Gd-DTPA-BMA (black) and with 5 mM Gd-DTPA-BMA (magenta). (L) Plots of the differences between the intensity reduction ratios observed for A2A AR in rHDL(DHA) and those observed for A2A AR in rHDL(ARA). The error bars represent the experimental errors, calculated from the root sum squares of the (noise level/signal intensity) in the four spectra, with and without Gd-DTPA-BMA in the experiments using A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA). (M) Distribution of the methionine residues in the crystal structure of A2A AR. The crystal structure of A2A AR with a full agonist, NECA (PDB code: 2YDV), is shown in ribbons in a side view, with the extracellular sides on the top. I106(M)^3.54 and A232(M)^6.34, which exhibited different solvent accessibilities between A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA), are depicted by purple sticks. M140^4.61, M211^5.72, and I292(M)^8.47, in which the solvent accessibilities of A2A AR in rHDL(DHA) were almost identical to those of A2A AR in rHDL(ARA), are depicted by dark gray sticks. M174, M177, M193, and M270, with resonances that were not observed in the presence of the full agonist, are depicted by gray sticks. NECA is depicted by gray sticks. The membrane position generated by the OPM database (http://opm.phar.umich.edu/) is indicated.
Fig. 6. Signaling activity–related conformational equilibria of A2A AR. (A to C) Correlation between the population of each conformation in the equilibrium and the G protein signaling. Plots of the [35S]-GTPγS binding rate to the complexes of A2A AR in rHDLs and a heterotrimeric G protein, consisting of human Gαs, bovine Gβ1, and human Gγ2, with the full agonist versus the normalized intensity of the M211A signal (A), and the normalized chemical shifts of M106 3.54  (B) and M232 6.34  (C). The [35S]-GTPγS binding rates and the errors are the same as those in Fig. 2B. The normalized chemical shifts and the errors were calculated in manners similar to those in Fig. 3F. (D) Schematic diagrams of the conformational equilibria of TM3, TM5, TM6, and TM7 of A2A AR in rHDLs. A2A AR in rHDL(POPC/POPG) bound to the inverse agonist-bound adopts an inactive NPxxYoff conformation. A2A AR in rHDL(POPC/POPG) bound to the partial agonist exists in equilibria between the NPxxYoff and multiple NPxxYon conformations, including the NPxxYon1 and NPxxYon2 conformations. The full agonist drives the equilibrium toward the NPxxYon conformations. The DHA chains redistribute the NPxxYon1 and NPxxYon2 conformations toward the NPxxYon2 conformation, which exhibits a large TM6 rotation angle and is more preferable for the G protein activation, as compared to the NPxxYon1 conformation.
by A2A AR and that the acyl chains derived from DHA did not affect the populations of the NPxxYoff and NPxxYon states in the presence of the partial agonist (Fig. 3, A to C). In contrast, the DHA chain induced a distribution change among the multiple NPxxYon substates (Figs. 3, D and E, and 4). The chemical shifts of M106 and M232 correlated well with the G protein activation rates observed for A2A AR in rHDL(POPC/POPG), A2A AR in rHDL(ARA), and A2A AR in rHDL(DHA) (Fig. 6, B and C), suggesting that the NPxxYon state includes the substates that are more preferable for the G protein activation than the other NPxxYon substates. Here, the former and latter NPxxYon substates are referred to as the NPxxYon2 and NPxxYon1 substates, respectively.

Chemical shift differences among A2A AR-rHDL(DHA), A2A AR-rHDL(ARA), and A2A AR-rHDL(POPC/POPG) were observed for M106 and M232, which exist at the cytoplasmic ends of TM3 and TM6 of A2A AR (Figs. 3 and 4), suggesting that the structures of these regions are affected by the type of surrounding lipids. In addition, the solvent PRE experiments revealed that the DHA chains shift the equilibrium of A2A AR toward the conformations with the higher solvent accessibility of M232 (Fig. 5). The crystal structures of A2A AR and other GPCRs indicated that the solvent accessibilities of the GPCR residues corresponding to M232 of A2A AR reflect the rotational angle of TM6 (Fig. S9, A and B, and see Supplementary Text). Our solvent PRE calculation of a model system, which exists in equilibrium between two conformations with different solvent accessibilities, suggested that the signal intensity reductions induced by Gd-DTPA-BMA, observed in the solvent PRE experiments, reflect the >30% population shift of the equilibrium among the conformations with >1 Å differences in the solvent accessibilities (Fig. S9, C and D, and see Supplementary Text) (27). In addition, the M232 signal of A2A AR/A232M in rHDL(DHA) was observed in the middle of those of A2A AR/A232M in rHDL(POPC/POPG) in the absence and presence of the G protein mimetic (Fig. S7E). The crystal structures of A2A AR (7, 9) revealed that the G protein mimetic induces large TM6 rotation by >25°. These results, along with the solvent PRE experiments, suggest that TM6 would undergo a large clockwise rotation, when viewed from the cytoplasmic surface, in the conformations in the NPxxYon2 substates (Fig. S9, E and F, and see Supplementary Text). The TM6 rotation of A2A AR would facilitate the association between A2A AR and the G protein, which could be the rate-limiting step in the activation of the G protein (29), because the TM6 rotation is required for the van der Waals interactions between the residues in TM6 of a GPCR and the C-terminal helix of the G protein, formed in the crystal structures of GPCRs bound to both a full agonist and a trimeric G protein or its mimetics (7, 11). Therefore, the NPxxYon2 conformation, which is highly populated in A2A AR-rHDL(DHA), would be preferable for the G protein activation.

On the basis of this structural interpretation of the resonances from M106 and M232, we propose the following signal regulation mechanism (Fig. 6D). A2A AR bound to the inverse agonist primarily adopts the inactive NPxxYoff conformation. In the presence of the full agonist, A2A AR exists in equilibria among the multiple active NPxxYon conformations, including the NPxxYon1 and NPxxYon2 conformations. The NPxxYon2 conformation adopts the large clockwise TM6 rotation and is more preferable for the G protein activation, as compared to the NPxxYon1 conformation. In the presence of the partial agonist, A2A AR exists in equilibrium between the aforementioned NPxxYoff and NPxxYon conformations. The DHA chains shift the equilibria among the NPxxYon conformations toward the NPxxYon2 conformation, which is more active than the NPxxYon1 conformation, leading to the enhanced G protein activation by A2A AR.

Mechanism underlying the population shift of the equilibria of A2A AR by the DHA chains

Accumulating evidence suggests that the conformations and activities of membrane proteins can be modulated by their interactions with lipids, locations in membrane domains, and the physical properties of the lipid bilayer (e.g., lipid order, membrane fluidity, curvature stress, and hydrophobic mismatch) (30). The location in the domains can be excluded from the mechanism underlying the effects of DHA observed in this study, because the purified A2A AR in rHDLs is isolated from other proteins. Whereas the average area per lipid, bilayer thickness, lateral diffusion, and lateral pressure profiles of the lipid bilayer with the ARA chains are reportedly similar to those with the DHA chains (31), remarkable differences were successfully observed for the G protein activation rates of A2A AR in rHDL(DHA) and A2A AR in rHDL(ARA) in this study. We also confirmed that the G protein activation by A2A AR in rHDLs was not affected by the addition of trifluoroethanol, which reportedly perturbs the lateral pressure profiles (table S1) (32). Thus, the changes in the physical properties of the bilayer by the DHA chains would have a limited effect on the function-related dynamics of A2A AR. In the recent 15-μs all-atom molecular dynamics simulation of A2A AR, the local lipid environment around A2A AR was significantly enriched with lipids containing unsaturated chains (33). Thus, it is tempting to speculate that the interaction between A2A AR and the DHA chains facilitates the clockwise rotation of TM6.

Relevance of stimulation of A2A AR by DHA

Although A2A AR in cells is embedded in membranes containing the DHA chains, information about the effects of DHA on the signaling activity of A2A AR was unavailable. In this study, the G protein activation rates of A2A AR in rHDL(DHA), A2A AR in rHDL(ARA), and A2A AR in rHDL(POPC/POPG) demonstrated that the DHA chains enhanced the G protein activation by A2A AR (Fig. 2B). In the lipid bilayers of the mammalian brain striatum, in which A2A AR is highly expressed (1), DHA constitutes 12 to 14% of the phospholipid acyl chains, and the population of the DHA chains is remarkably decreased to 3 to 8% by feeding DHA-deficient diets (3, 4). In our experiment, the initial [35S]-GTPγS binding rate of A2A AR in rHDL(DHA), in which DHA constitutes 15% of the acyl chains, was 1.29 ± 0.03 times higher than that of A2A AR in rHDL(POPC/POPG) (n = 6). The effect of the DHA chains is higher than the level observed for the partial agonist (~10%; Fig. 1A), suggesting that the DHA chains in cell membranes play an indispensable role in the activation of A2A AR.

Effects of various lipids on the conformation and activities of GPCRs

Cholesterol, anionic lipids, and other lipids, as well as the DHA chains, reportedly influence the GPCR activities (21). The compositions of biological membranes are affected by various pathological conditions, including aging, metabolic disease, Alzheimer’s disease, and cancer. Thus, knowledge about the mechanisms underlying the influences of lipids on the modulation of GPCR activities is helpful for understanding the GPCR functions under normal and pathological conditions. The effects of lipids on the signaling activities and...
the conformations of GPCRs may be different among various GPCRs and lipids, because the residues in the TM region directed toward the lipids are not conserved among class A GPCRs. For example, a previous ultraviolet–visible absorption spectroscopic study of rhodopsin indicated that the DHA chains facilitate the transition from metarhodopsin I to metarhodopsin II, which reportedly adopt conformations containing the inactive and active NPxxY motifs, respectively (34), whereas the populations of the NPxxYoff and NPxxYon conformations of A2A AR were not affected by the DHA chains (Fig. 3, A to C). The methyl resonances of deuterated GPCRs embedded in rHDLs with various lipid compositions are widely applicable for analyses of the effects of lipids on the function-related dynamics of GPCRs. Solid-state NMR has been used for studying lipid membrane protein interactions and conformations of membrane proteins embedded in lipid bilayer environments (35). Thus, solid-state NMR, which allows flexibility with respect to the lipid preparations, NMR detection schemes, and accessible temperature range, will be valuable for studying GPCRs in lipid bilayers.

Our studies using A2A AR in rHDL lipid bilayers revealed that the DHA chains enhanced the G protein activation by A2A AR. To our knowledge, this is the first report demonstrating the activation of A2A AR by DHA. In addition, our NMR studies of A2A AR in rHDL lipid bilayers with various lipid compositions are widely applicable for analyses of the effects of lipids on the function-related dynamics of GPCRs. Solid-state NMR has been used for studying lipid membrane protein interactions and conformations of membrane proteins embedded in lipid bilayer environments (35). Thus, solid-state NMR, which allows flexibility with respect to the lipid preparations, NMR detection schemes, and accessible temperature range, will be valuable for studying GPCRs in lipid bilayers.

MATERIALS AND METHODS
Statistics and general methods
No statistical methods were used to predetermine the sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiment and outcome assessment.

Reagents
All reagents were purchased from Nacalai Tesque or Wako Pure Chemicals, unless otherwise noted. The [α,β,β-2H, methyl-13C]-1-methionine was synthesized by the enzymatic deuteration of [methyl-13C]-1-methionine (ISOTEC or Cambridge Isotope Laboratories) with Escherichia coli cystathionine γ-synthase, as previously described (36). The xanthine amine congener (XAC)-agarose gel was prepared as reported previously (37).

Expression and purification of A2A AR
The complementary DNA fragment encoding human A2A AR (1 to 316), with a different secretion signal (α-factor) and a C-terminal 10× His-tag, was amplified by overlap extension polymerase chain reaction and cloned into the pPICZαA vector (Thermo Fisher Scientific) via the Eco RI–Sal I sites. Mutations were introduced with a QuikChange site-directed mutagenesis kit (Stratagene). The M1A/M4T mutation was introduced in all constructs, unless otherwise stated. The pPICZαA vector containing the A2A AR gene was linearized with Pme I and purified with a QIAquick nucleotide extraction kit (Qiagen). The linearized vector was transformed into P. pastoris X-33 cells by electroporation.

For the expression of [[α,β,β-2H, methyl-13C] Met, u-2H] A2A AR, the cells were initially grown in 10 ml of 2H-buffered minimal glycercol (BMG) medium [100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4 × 10−3% biotin, 1% (v/v) glycerol, and H2O/D2O = 5/95], supplemented with zeocin (100 μg/ml), at 29°C for 2 to 3 days, with shaking at 230 revolutions per minute (rpm). The cells were then transferred to 1 liter of H-BM medium, and the culture was incubated in a bioreactor vessel (Takasugi Co. Ltd., Tokyo, Japan) at 29°C, with shaking at 350 rpm. During this culture period, the pH value of the culture was maintained at 5.8 by adding a sodium hydroxide solution. After 2 days, 5 ml of glycerol was added, and the culture was continued for 26 hours. The cells were then centrifuged at 3000g and resuspended in H2-buffered minimal methanol (BMM) medium [100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 4 × 10−3% biotin, 0.5% methanol-d4 (Cambridge Isotope Laboratories), and H2O/D2O = 5/95], supplemented with 10 mM theophylline, lysine (800 mg/liter), threonine (400 mg/liter), isoleucine (200 mg/liter, and [α,β,β-2H, methyl-13C]-methionine (600 mg/liter). The cells were grown at 22°C, with shaking at 350 rpm for 20 hours, and 2.5 ml methanol-d4 was added. The incubation was continued for 40 hours, and the cells were harvested by centrifugation at 9000g.

All of the following procedures were either performed on ice or at 4°C. The cells were resuspended in buffer A [50 mM tris-HCl (pH 7.4), 200 mM NaCl, 5 mM EDTA, 0.2 mM 4-[(2-aminoethyl)benzenesulfonyl] fluoride hydrochloride, 20 μM leupeptin hemi-sulfate (Peptide Institute Inc.), 28 μM E-64, 0.6 μM aprotinin, and 30 μM pepstatin A (Peptide Institute Inc.)] and disrupted by agitation at 300 rpm at 4°C using 40 ml zirconia/silica beads (BioSpec Products). Glycerol was added to the cell lysate, at a final concentration of 10% (v/v). The cell lysate was centrifuged at 10,500g for 10 min, and the resulting supernatant was centrifuged at 140,000g for 60 min. The membrane pellet was suspended in 5 ml of buffer B [50 mM tris-HCl (pH 8.0), 200 mM NaCl, and 10% (v/v) glycerol], supplemented with 10 mM theophylline, and was stored at −80°C. The membranes were solubilized in 2 to 3 ml of buffer B, supplemented with 10% DDM and 10 mM theophylline, for 4 hours, and were then centrifuged at 150,000g for 30 min. The supernatant was batch incubated overnight with 1 ml of TALON metal affinity resin (Clontech). The resin was first washed with 15 ml of buffer B, supplemented with 0.05% DDM, 10 mM theophylline, and 5 mM imidazole, and subsequently washed with 15 ml of buffer B, supplemented with 0.05% DDM and 5 mM imidazole. The protein was eluted with 15 ml of buffer B, supplemented with 0.05% DDM and 250 mM imidazole. The eluate was batch incubated for 5 hours with 1 ml of XAC-agarose gel. The resin was washed with 5 ml of buffer B, supplemented with 0.05% DDM, and the protein was eluted with 16 ml of buffer B, supplemented with 0.05% DDM and 30 mM theophylline. The eluted A2A AR was concentrated to ~1 mg/ml, using a centrifugal filter device (Amicon Ultra-15, 30 kDa molecular weight cutoff, Millipore), and was stored at −80°C.

Preparation of A2A AR in rHDLs
MSP1E3 was expressed and purified as previously described (20), with minor modifications, as follows. The double-stranded DNA encoding the N-terminal 7× His-tag, tobacco etch virus (TEV) protease recognition site, and MSP1E3 gene (19) was transferred into the pET43a vector (Novagen), with a modification of the nucleotides around the Psh AI site from 5′-GACAAGAGTCCGGGAGC-3′ to 5′-GACAAGTGTCCGGGCTTCTCCTCAACGATATCT-3′, using T4 DNA polymerase and T4 DNA ligase. The gene
encoding the NUS tag was removed by Nde I digestion and subsequent ligation, and a gene encoding the C-terminal ID4 tag was inserted with the QuikChange site-directed mutagenesis kit. The plasmid for MSP1 expression was constructed in a similar manner, except for the absence of the C-terminal ID4 tag. The E. coli BL21 (DE3) cells were transformed with the plasmid and cultured in Terrific Broth medium at 37°C. Protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, when the optical density at 600 nm (OD600) reached 2.5. After 3 hours of induction, the cells were harvested and sonicated in buffer containing 20 mM tris-HCl (pH 8.0), 300 mM NaCl, 0.1 mM 4-(2-aminoethyl) benzenesulfonylamide, and protease inhibitor cocktail (Nacalai Tesque). The cell lysate was centrifuged at 20,000g for 30 min, and the precipitate was solubilized in buffer containing 1% (v/v) Triton X-100. After centrifugation at 100,000g for 1 hour, the supernatant was batch incubated for 2 hours with 10 ml of HIS-Select resin (Sigma-Aldrich). The resin was washed with 500 ml of buffer containing 1% (v/v) Triton X-100, followed by 500 ml of buffer containing 10 mM sodium cholate and 100 ml of buffer containing 10 mM imidazole. The protein was eluted by 50 ml of buffer containing 300 mM imidazole and was dialyzed against buffer containing 50 mM NaPi (pH 8.0), 200 mM NaCl, and 10% (v/v) glycerol. The His-tag was cleaved by TEV protease and removed by passage through HIS-Select resin.

Solutions of POPC, POPG, SDPC, SDPG, SAPC, DDPC, and DDPG in chloroform (Avanti Polar Lipids) were mixed at molar ratios of POPC:POPG = 3:1, SDPC:SDPG:POPC:POPG = 9:3:3:1, SAPC:POPG = 3:1, SDPC:POPG = 3:1, DDPC:POPG:DDPC = 9:15:3:5, and SDPC:POPG:DDPC:POPG = 9:21:3:7. The solvent was evaporated under a nitrogen atmosphere and dried in vacuo, to form a lipid film. The film was solubilized by the addition of buffer C (50 mM tris-HCl (pH 8.0) and 200 mM NaCl), supplemented with 200 mM sodium cholate, for a final lipid concentration of 50 mM.

All of the following procedures were performed either on ice or at 4°C. The purified A2A AR in DDM micelles (30 nmol), MSP1E3, and lipids were mixed at a final molar ratio of 1:10:120 in 1.5 ml of buffer C and incubated for 1 hour. To remove the detergents, 80% (w/v) of Bio-Beads SM-2 (Bio-Rad) was added to the solution and incubated for 3 hours with gentle mixing. The supernatant was batch incubated for 2 hours with 0.25 ml of TALON resin. The resin was washed with 1.25 ml of buffer C, supplemented with 5 mM imidazole. The A2A AR in rHDLs was eluted from the resin with 1.5 ml of buffer C, supplemented with 250 mM imidazole. The eluate was further purified by size exclusion chromatography on a Superdex 200 10/30 GL column, eluted with buffer C.

For the NMR experiments, the purified A2A AR in rHDLs was concentrated with a centrifugal filter device (Amicon Ultra-4, 30 kDa molecular weight cutoff, Millipore), with simultaneous buffer exchange to buffer D [20 mM sodium phosphate (pH 7.0) and H2O/D2O = 1/99], supplemented with and without the ligands [100 μM NECA, regadenoson, ZM241385, or 25 μM LF5834 (Tocris)]. For the solvent PRE experiments, Gd-DTPA-BMA (Omniscan, Daichii Sankyo) was added to the sample at a final concentration of 5 mM. The added peak was removed by TEV protease and removed by passage through HIS-Select resin.

Radioligand-binding assays
The NECA saturation binding assays were performed by incubating the purified A2A AR in rHDLs with five different concentrations of [3H]-NECA (PerkinElmer), between 1 nM and 0.5 μM, in 100 μl of buffer E [50 mM tris-HCl (pH 8.0)]. The competition binding assays between [3H]-NECA and LF5834 were performed by incubating the purified A2A AR in rHDLs with 50 nM of [3H]-NECA and eight different concentrations of LF5834, between 0.1 and 1 μM, in 100 μl of buffer E. The competition binding assays between [3H]-NECA and the nonlabeled NECA were performed by incubating the purified A2A AR in rHDLs with 50 nM of [3H]-NECA and 12 different concentrations of NECA, between 0.1 and 10 mM, in 100 μl of buffer E. After a 1-hour incubation on ice, the solution was applied to a NAP-5 size exclusion column (GE Healthcare). The fractions containing A2A AR were mixed with Optiphase Supermix (PerkinElmer), and the radioactivity was detected with a liquid scintillation counter (MicroBeta2, PerkinElmer). Nonspecific binding was assessed by performing identical reactions with an excess amount of ZM241385.

The ZM241385 saturation binding assays were performed by incubations of the membrane precipitate containing expressed A2A AR with 0.3, 1, and 3 nM [3H]-ZM241385 (American Radiolabeled Chemicals) in 200 μl of buffer E. After a 1-hour incubation at 25°C on an orbital shaker (200 rpm), the solution was passed through a UniFilter-96 GF/C (PerkinElmer) by a cell harvester (PerkinElmer). After a 1-hour incubation at 50°C, MicroScint (PerkinElmer) was added to the filter, and the radioactivity was detected with a liquid scintillation counter.

Preparation of the G protein and its mimetic
Human Gαs was prepared as previously described (18), with slight modifications, as follows. Double-stranded DNA encoding the N-terminal hexahistidine tag, thrombin recognition site, and human Gαs gene was transferred into the pET15b vector (Novagen). The E. coli BL21 (DE3) cells were transformed with the plasmid and cultured in LB medium at 37°C. Protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, when the OD600 reached 0.6 to 0.7. The cells were cultivated at 18°C for 6 hours after induction. After centrifugation at 9000g for 10 min, the cells were resuspended in buffer B [20 mM tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM MgCl2, and 0.1 mM GDP] and stored at −80°C. The cells were lysed by sonication and centrifuged at 150,000g for 10 min, the supernatant was centrifuged at 9000g for 10 min, and the resulting supernatant was dialyzed against buffer C [50 mM tris-HCl (pH 8.0) and 200 mM NaCl], supplemented with 200 mM sodium cholate, for a final lipid concentration of 100 mM.

All of the following procedures were performed either on ice or at 4°C. The purified Gαs was transferred into the pET15b vector for MSP1 expression was constructed in a similar manner, except for the absence of the C-terminal ID4 tag. The E. coli BL21 (DE3) cells were transformed with the plasmid and cultured in Terrific Broth medium at 37°C. Protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, when the OD600 reached 0.6 to 0.7. The cells were cultivated at 18°C for 6 hours after induction. After centrifugation at 9000g for 10 min, the cells were resuspended in buffer B [20 mM tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM MgCl2, and 0.1 mM GDP] and stored at −80°C. The cells were lysed by sonication and centrifuged at 150,000g for 30 min. NaCl was added to the resulting supernatant at a final concentration of 100 mM, and the solution was batch incubated for 2 hours with 5 ml of Complete His-tag purification resin (Roche), equilibrated with buffer B. The resin was washed with 50 ml of buffer B containing 500 mM NaCl, followed by 100 ml of buffer B containing 100 mM NaCl and 20 mM imidazole. The protein was eluted by 100 ml of buffer B containing 100 mM NaCl and 150 mM imidazole. The eluate was applied to 1 ml of HitTrap Q HP (GE Healthcare), equilibrated with buffer B. The protein was eluted by a linear concentration gradient of NaCl, from 0 to 500 mM, and the eluate was concentrated with a centrifugal filter device (Amicon Ultra-15, 30 kDa molecular weight cutoff) with 300 mM NaCl. The buffer was exchanged to 20 mM tris-HCl (pH 8.0), 2 mM MgCl2, 0.5 mM EDTA, 20 μM GDP, 0.1 mM PMSF, and 10% (v/v) glycerol, and the sample was stored at −80°C. The heterotrimeric G protein consisting of human Gαs, bovine Gβ1, and human Gγ2 was prepared, as previously described (19).

The G protein mimetic was prepared as previously, with modifications as follows. The DNA fragment encoding the engineered Gαs gene (mini-Gs) was cloned into the above-described plasmid for the expression of MSP1, which contains the N-terminal 7× His-tag and the TEV protease recognition site, with an In-Fusion HD cloning kit (Clontech). The E. coli BL21 (DE3) codon plus RP cells were
transformed with the plasmid and cultured in LB medium at 37°C. Protein expression was induced by the addition of 50 μM isopropyl 1-thio-β-D-galactopyranoside, when the OD_{600} reached 0.6 to 0.7. The cells were cultivated at 25°C for 20 hours after induction. After centrifugation at 9000g for 15 min, the cells were resuspended in a buffer containing 40 mM Hepes (pH 7.5), 100 mM NaCl, 10% glycerol, 10 mM imidazole, 5 mM MgCl₂, 50 μM GDP, 1 mM 4-[(aminoethyl)benzenesulfonamido]fluoride hydrochloride, 1.5 μM pepstatin A, 20 μM leupeptin, and 100 μM diithiothreitol and stored at −80°C. The cells were lysed by sonication and centrifuged at 100,000g at −80°C. The supernatant was added to the 10 ml of HIS-Select resin, and the flow-through was collected and added to the column. The column was washed with 200 ml of buffer G [20 mM Hepes (pH 7.5), 10% glycerol, 1 mM MgCl₂, and 50 μM GDP] containing 40 mM imidazole and 500 mM NaCl, followed by 100 ml of buffer G containing 40 mM imidazole and 100 mM NaCl. The protein was eluted by 30 ml of a buffer G containing 500 mM imidazole and 100 mM NaCl. TEV protease was added to a final ratio of 1:20 w/w (TEV-mini-Gs) and incubated for 18 hours at room temperature. The sample was dialysed overnight against 2 liters of buffer G containing 100 mM NaCl and added to the 8 ml of HIS-Select resin. The flow-through was collected and passed through the column five times and was concentrated with a centrifugal filter device (Amicon Ultra-15, 10 kDa molecular weight cutoff). The protein was further purified by size exclusion chromatography on a HiLoad 26/60 Superdex prep grade column and eluted with buffer containing 10 mM Hepes (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM MgCl₂, 1 μM GDP, and 0.1 mM tris(2-carboxyethyl)phosphine.

[35S]-GTPγS binding assays
A₂AAR in rHDLs and the G protein were mixed at final concentrations of 20 and 50 nM, respectively, in 100 μl of buffer F [50 mM tris-HCl (pH 8.0), 5 mM MgCl₂, and 1 μM GDP], supplemented with or without ligand (100 μM NECA, 100 μM regadenoson, 100 μM ZM241385, or 25 μM LUF5834). The GDP/GTPγS exchange was initiated by the addition of [35S]-GTPγS, at a final concentration of 10 nM. After a 30-min incubation at 25°C, the reaction mixture was applied to a NAP-5 size exclusion column. The fractions containing the G protein were mixed with Optiphase Supernix, and the radioactivity was detected with the liquid scintillation counter.

NMR experiments
The [1H-13C] HMBC and one-dimensional 1H spectra of A₂AAR in rHDLs and in DDM micelles were recorded with a Bruker Avance 800 spectrometer equipped with a cryogenic probe. The one-dimensional 31P spectra of A₂AAR in rHDLs were recorded with a Bruker Avance 400 spectrometer equipped with a broadband/19F observe probe. 1H-13C HMBC spectra were recorded for 15 to 40 μM [[α,β,β-2H, methyl-13C]Met, u-2H] A₂AAR or its mutants, in rHDLs and in DDM micelles in buffer D. In the NMR experiments with A₂AAR in DDM micelles, 0.19% DDM and 0.038% cholesteryl hemisuccinate were added to the samples. In the NMR experiments with A₂AAR in the UK432097-, NECA-, regadenoson-, ZM241385-, and LUF5834-bound states, UK432097, NECA, regadenoson, ZM241385, and LUF5834 were added to the samples at final concentrations of 25, 100, 100, 100, and 25 μM, respectively. In the solvent PRE experiments, Gd-DTPA-BMA was added to the samples at a final concentration of 5 mM. In the NMR experiments of the ternary complex, the engineered G protein was added to the sample with 15 μM A₂AAR/A232M in the NECA-bound state at a final concentration of 30 μM. The spectral widths were set to 12,800 Hz and 6400 to 7400 Hz for the 1H and 13C dimensions, respectively, and the interscan delays were set to 1 s. In total, 512 × 128 complex points were recorded, and 256 scans/free induction decay gave rise to an acquisition time of 20 to 23 hours for each spectrum. The spectra were referenced to 3-(trimethylsiloxy)-1-propanesulfonic acid sodium salt, in both the 1H and 13C dimensions. One-dimensional 1H NMR spectra were recorded with a repetition period of 1.0 s, with 8096 complex data points and a spectral width of 12,800 Hz. One-dimensional 31P NMR spectra were recorded with a repetition period of 1.0 s, with 2048 complex data points and a spectral width of 3240 Hz. The assignments of the resonances from the DHA and ARA chains were derived from the previous report (38). All of the spectra were processed and analyzed by the Topspin 2.1, 3.1, or 3.5 software (Bruker).

In situ chemical shift error analysis
The calculations were performed by in-house developed programs, written in the Python 2.7 programming language, supplemented with the extension modules NumPy 1.15, SciPy 1.1, and Cython 0.29. Synthetic two-dimensional time domain data, composed of 100 synthetic signals with relaxation rates and signal-to-noise ratios similar to those of the observed resonances, were generated using in-house developed programs. The data were processed in the same manner as the observed data, and the SDs of the chemical shifts were calculated.

GPCR structure database analysis
In total, 182 coordinate files of GPCR structures were obtained from Protein Data Bank (PDB), and the structures without residues corresponding to "5.63" or "6.27" in the GPCRdb numbering scheme (25) were excluded from the analysis, because the solvent accessibilities were overestimated for these structures. For the remaining 118 structures, the lipid bilayer was added by CHARMM-GUI Membrane Builder (39).

In each structure, hypothetical atoms with a 3.5-Å radius, corresponding to Gd-DTPA-DMA, were evenly placed at grid points spaced 1 Å surrounding GPCR, and the hypothetical atoms in which at least one of the GPCR and/or lipid atoms are within the sum of the radii were removed. The distances between the closest hypothetical atom and the Cβ atom of the residues corresponding to M106 and M232 of A₂AAR were used for the calculation of the solvent PRE, based on the Hwang-Freed model, as reported previously (27). The full crowding effect was not implemented, considering the low concentration of A₂AAR in rHDLs, whereas the correlated motion of Gd-DTPA-BMA and A₂AAR in rHDLs were taken into account by setting the diffusion coefficient of Gd-DTPA-BMA equal to that of A₂AAR in rHDL (approach B) (27). The hydrodynamic radius of A₂AAR in rHDLs was set to 6 nm.

The TM3 to TM6 distances are the distances between the mean coordinates of the main-chain atoms of “3.49,” “3.50,” “3.51,” “3.52,” “3.53,” and “3.54” residues (GPCRdb numbering scheme) (25) and those of the “6.31,” “6.32,” “6.33,” “6.34,” “6.35,” and “6.36” residues. The TM6 rotation angles are the angles between the vectors connecting the mean coordinates of the main-chain atoms of the “1.36,” “1.37,” “1.38,” “1.39,” “1.40,” “1.41,” “2.57,” “2.58,” “2.59,” “2.60,” “2.61,” “4.53,” “4.54,” “4.55,” and “4.56” residues and those of the “1.52,” “1.53,” “1.54,” “1.55,” “1.56,” “2.40,” “2.41,” “2.42,” “2.43,” “2.44,” “4.44,” “4.45,” and “4.47” residues and the vectors connecting the mean coordinates of the Cα and Cβ atoms of the “6.34” residue.
**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/12/eaay8544/DC1

**Acknowledgments**

This work is supported by The Ministry of Education, Culture, Sports, Science and Technology (MEXT)/Japan Society for the Promotion of Science (JSPS) KAKENHI grant numbers JP17H06097, JP18H01353, JP19H05490, and JP17J1420; the development of core technologies for innovative drug development based upon IT and the development of innovative drug discovery technologies for middle-sized molecules, from the Japan Agency for Medical Research and Development, AMED (to I.S.); M.N. is a JSPS fellow. **Author contributions:** T.M., K.K., Y.K., Y.T., T.S., and I.S. designed the research. T.M., K.K., M.K., Y.K., M.N., S.I., Y.S., and T.U. performed the research. T.M., K.K., M.K., Y.K., S.I., Y.S., T.U., and I.S. analyzed the data. T.M., K.K., Y.K., T.S., and I.S. wrote the manuscript. Competing interests: The authors declare that they have no competing interests.

Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

Submitted 23 July 2019
Accepted 18 December 2019
Published 18 March 2020

10.1126/sciadv.aay8544

**Citation:** T. Mizumura, K. Kondo, M. Kurita, Y. Kofuku, M. Natsume, S. Imai, Y. Shiraishi, T. Ueda, I. Shimada, Activation of adenylate receptor 2 by lipids from docosahexaenoic acid revealed by NMR spectroscopy. *Adv. Sci.*, 6, eaay8544 (2020).