Many drugs that target G-protein-coupled receptors (GPCRs) induce or inhibit their signal transduction with different strengths, which affect their therapeutic properties. However, the mechanism underlying the differences in the signalling levels is still not clear, although several structures of GPCRs complexed with ligands determined by X-ray crystallography are available. Here we utilized NMR to monitor the signals from the methionine residue at position 82 in neutral antagonist- and partial agonist-bound states of β2-adrenergic receptor (β2AR), which are correlated with the conformational changes of the transmembrane regions upon activation. We show that this residue exists in a conformational equilibrium between the inverse agonist-bound states and the full agonist-bound state, and the population of the latter reflects the signal transduction level in each ligand-bound state. These findings provide insights into the multi-level signalling of β2AR and other GPCRs, including the basal activity, and the mechanism of signal transduction mediated by GPCRs.
-protein-coupled receptors (GPCRs) are one of the largest membrane protein families in eukaryotes, and more than 25% of modern drugs target GPCRs. These drugs bind to GPCRs, leading to the induction or inhibition of signal transduction mediated by G-proteins, β-arrestins and various other effectors via GPCRs. Each chemical ligand for a GPCR has a different level of ability to activate or inhibit its target, which is commonly referred to as efficacy, and the ligands are classified according to their efficacies, such as full agonists, partial agonists, neutral antagonists and inverse agonists. These differences in the efficacies significantly affect the therapeutic properties of the GPCR ligands. In the case of drugs that target β2-adrenergic receptor (β2AR), a full agonist offers a clinical advantage over a partial agonist in acute severe asthma, although full agonists are capable of causing more adverse effects.

Structural analyses of β2AR complexed with various ligands are required to clarify the mechanism that determines the ligand efficacies. The crystal structures of β2AR have been solved in the forms bound to inverse agonists, a neutral antagonist, a full agonist, a protein-mimicking nanobody or a G-protein-mimicking receptor (PDB accession codes: 3D4S, 2RIH, 3PDF, 3SN6, and 3POG). In the structure of the form bound to both a full agonist and a G-protein, the cytoplasmic half of transmembrane 6 (TM6) shifted outward, in comparison with that in the inverse agonist-bound form, and the C-terminal helix of the G-protein was inserted into a cytoplasmic cavity. In addition, the ligand-dependent conformational changes of the solvent-exposed extracellular and intracellular regions were observed in biochemical studies with fluorescent labeling, chemical labeling and hydrogen–deuterium exchange techniques, as well as solution NMR analyses using chemical modifications with 13CH3 probes. However, the structure of the β2AR TM region, which is directly involved in the conformational changes upon ligand binding, has not been examined in the neutral antagonist-bound and partial agonist-bound states. In addition, the crystal structures cannot fully explain the mechanism of the efficacies, including the basal activity, which is more exposed to solvent in I than in A, and the population of A correlates with the efficacy of each ligand. I and A were structurally characterized by the PRE experiments, which suggested that C32754 is more exposed to solvent in I than in A, in an agonist-bound state.

Here we utilized NMR to clarify the conformational diversity of the TM region of β2AR in the inverse agonist-bound state, neutral antagonist-bound state, partial agonist-bound state and full agonist-bound state. Our NMR analyses revealed that the methionine residue at position 82 is in a conformational equilibrium between the inverse agonist-bound states and the full agonist-bound state, and the population of the latter reflects the signal transduction level in each ligand-bound state.

Results

Preparation and characterization of β2AR. The E122W/N187F/C265A mutant of β2AR (Gly2-Gly365) with an N-terminal FLAG-tag and a C-terminal decahistidine-tag, which exhibited agonist-dependent signalling activities almost identical to the wild type in previous studies, was expressed in a baculovirus–insect cell expression system. β2AR was solubilized by n-dodecyl-β-D-maltopyranoside (DDM), and purified by three chromatography steps, including ligand-affinity chromatography, to more than 95% purity, as judged from SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analyses. The purified β2AR is in the formoterol-bound state, because formoterol was added during the elution from the ligand-affinity chromatography. Radioligand-binding assays with an excess amount of [3H]-dihydroalprenolol (DHA), which has a higher affinity for β2AR than formoterol, revealed that more than 80% of the purified β2AR retained the DHA-binding activity. We also confirmed that the purified β2AR exhibited ligand-dependent conformational changes, by experiments using a fluorescent probe introduced at C265 (Fig. 1a,b).

Methionine residues are frequently observed in TM3, TM5, and TM6 of GPCR, and these regions exhibit large conformational changes upon activation (Supplementary Fig. S1). β2AR possesses nine methionine residues in extracellular loop 1 (ECL1), TM1, TM2, TM4, TM5 and TM6 (Fig. 2 and Table 1), and M82 and M215 (Supplementary Figs S2 and S3), M215 and M279 (Supplementary Figs S2 and S3), M215 and M279 assume distinctly different conformations between the inverse agonist-bound and the full agonist/G-protein-bound crystal structures. Therefore, we utilized the methionine methyl groups to investigate the conformation of the TM region of β2AR in various ligand-bound states. Methionine methyl-selective 13C labeling in the baculovirus–insect cell expression system was accomplished by adding [methyl-13C]methionine to methionine-deficient medium. We confirmed that about 90% of the methionine methyl groups in thioredoxin, prepared by the same procedure for β2AR, were labeled with 13C and that the other types of amino-acid residues were not significantly labeled. 1H-13C heteronuclear multiple quantum coherence (HMQC) spectra of the [methyl-13C-Met] β2AR in the formoterol-bound state were recorded, and those in the carazolol-bound state were recorded by adding an excess amount of carazolol.

![Figure 1 | Analyses of ligand-dependent conformational changes in β2AR with fluorescent probes.](image-url)
For the assignments of the resonances from the methionine in the TM region, we introduced a further mutation into the methionine residue of interest (Supplementary Fig. S5). For example, M82V\(^{2.53}\) was assigned by introducing the M82V mutation into the 4Met mutant (Supplementary Fig. S5d). We confirmed that M82V retains the native folding in both the carazolol- and formoterol-bound states, by experiments using a fluorescent probe introduced at C265\(^{2.27}\) (Fig. 1b and Supplementary Fig. S6). In addition, the M82V mutation does not affect the affinity of the antagonist \(^{[3]}\)H DHA to \(\beta_2\)AR (Supplementary Fig. S7). In the carazolol-bound state, two signals were absent in the spectrum of the M82V mutant, revealing that both of these resonances are from M82\(^{2.53}\) (Fig. 3c,e). Hereafter, we refer to the downfield and upfield resonances from M82\(^{2.53}\) as M82\(^{D}\) and M82\(^{U}\), respectively. In the formoterol-bound state, one signal was absent in the spectrum of the M82V mutant, revealing that this resonance is from M82\(^{2.53}\) (Fig. 3d,f). The chemical shifts of the resonance from M82\(^{2.53}\) in the formoterol-bound state were different from M82\(^{D}\) and M82\(^{U}\) in the carazolol-bound state (Fig. 4a). Hereafter, this resonance from M82\(^{2.53}\) in the full agonist formoterol-bound state is referred to as M82\(^{E}\).

The resonances from M215\(^{5.54}\) and M279\(^{6.41}\) in the carazolol-bound state were also assigned using the 4Met-based mutants (Supplementary Fig. S5e,f). The spectra of the mutants for M215\(^{5.54}\) and M279\(^{6.41}\) in the formoterol-bound state were not significantly different from those without these mutations, suggesting that the resonances from M215\(^{5.54}\) and M279\(^{6.41}\) were not observed in the formoterol-bound state (Fig. 4a).

**M82 in the states bound with antagonist and partial agonists.** To investigate the structures of the TM region in the neutral antagonist-bound and partial agonist-bound states, the HMQC spectra of the 4Met mutant labeled with methyl-\(^{13}\)C-Met were recorded in the state bound with the neutral antagonist, alprenolol, and in those bound with the partial agonists, tulobuterol and clenbuterol (Fig. 4b,c). Alprenolol does not alter the basal activity of \(\beta_2\)AR\(^{16,19}\). Clenbuterol reportedly has higher efficacy than tulobuterol\(^{24}\) (Supplementary Table S1). The tulobuterol- and clenbuterol-bound states were achieved by extensive washing with these ligands on cobalt-affinity resin after the ligand-affinity purification steps. The alprenol-bound state was attained by adding an excess amount of alprenolol over clenbuterol. As a result, in the alprenol-bound state, a major and a minor resonance that slightly shifted from M82\(^{U}\) and M82\(^{D}\), respectively, were observed (Fig. 4b,c). In both the tulobuterol- and clenbuterol-bound states, a signal was observed at a chemical shift between M82\(^{U}\) and M82\(^{A}\), and the chemical shifts in the tulobuterol-bound state were closer to those of M82\(^{U}\) (Fig. 4b,c).

To examine whether the resonances from M82\(^{2.53}\) in the ligand-bound states undergo conformational exchange, we also recorded the spectra at a lower temperature, 283 K (Fig. 4d and Supplementary Fig. S8). As a result, the resonances from M82\(^{2.53}\) in the tulobuterol- and clenbuterol-bound states significantly shifted away from M82\(^{U}\), and the M82\(^{A}\) resonance in the formoterol-bound state slightly shifted away from M82\(^{U}\) at 283 K (Fig. 4d and Supplementary Fig. S8a–c), whereas the resonances from the methyl groups of tulobuterol and clenbuterol did not shift (Supplementary Fig. S8d,e). In the spectra of \(\beta_2\)AR with lower concentrations of clenbuterol and tulobuterol, the signals were observed at the same chemical shifts as those at higher ligand concentrations (Supplementary Fig. S9), suggesting that the NMR signals are not significantly affected by the exchange between the free and bound states or non-specific effect of the ligands, because \(\beta_2\)AR is only in the ligand-bound state under the present solution conditions. This is consistent with the estimation of the bound population of \(\beta_2\)AR by the ligand concentration of the NMR samples and the reported affinities of the ligands (Supplementary Table S1).
The resonances from M82 are closer to those in the carazolol-bound state. The intensities of residues except for M82 were also closer to those in the carazolol-bound state. The chemical shifts in the alprenolol-bound state and the chemical shifts in the alprenolol-bound state were between those in the carazolol- and clenbuterol-bound states. The chemical shifts from M40 in the carazolol-bound state, and M40, M215 and M279 in the formoterol-bound states are shown in Fig. 5a. The regions with methionine chemical shifts are shown, and the assigned resonances are indicated. The resonances from M40 in the carazolol-bound state, and M40, M215 and M279 in the formoterol-bound state were not observed. Resonances indicated with single asterisks are derived from minor impurity proteins from insect cell membranes, but not from β2-AR. Double asterisks are from the intense DDm signal with an ω1 chemical shift of 1.6–1.7 p.p.m.

Signals from the residues except for M82. The resonances from M215, which is in the intracellular side of TM5, in the states with carazolol, alprenolol, tulobuterol and formoterol are shown in Fig. 5a,b. M215 exhibited single resonance in the carazolol-, alprenolol-, tulobuterol- and clenbuterol-bound states (Fig. 5a,b). The chemical shifts in the alprenolol- and tulobuterol-bound states were between those in the carazolol- and clenbuterol-bound states, and the chemical shifts in the alprenolol-bound state were closer to those in the carazolol-bound state. The intensities were in the following order: carazolol-bound state > alprenolol-bound state > tulobuterol-bound state > clenbuterol-bound state and the resonances from M215 were not observed in the formoterol-bound state (Table 1). As the temperature was lowered from 298 K to 283 K, the M215 resonances in the carazolol- and alprenolol-bound states shifted towards that in the carazolol-bound state (Supplementary Fig. S10). The chemical shifts and intensities of the resonances from M279, which is in the intracellular side of TM6, also exhibited similar bound ligand-dependent changes (Fig. 5c).

### Table 1 | Summary of the differences in β2-AR methionine resonances in the states with various efficacies.

| Position in the β2-AR structure | M36 | M40 | M82 | M96 | M98 | M156 | M171 | M215 | M279 |
|----------------------------------|-----|-----|-----|-----|-----|------|------|------|------|
| Structural element*              | TM1 | TM1 | TM2 | TM2 | ECL1| TM4  | TM4  | TM5  | TM6  |
| Extracellular half (E) or intracellular half (I) | E   | E   | E   | E   | E   | E    | I    | I    | I    |
| Large conformational change upon activation | No  | No  | Yes | No  | No  | No   | No   | Yes  | Yes  |

### Number of observed resonances in each ligand-bound state

| Ligand     | Carazolol | Alprenolol | Tulobuterol | Clenbuterol | Formoterol |
|------------|-----------|------------|-------------|-------------|------------|
| Resonances | 1         | 2          | 1           | 1           | 1          |
| M215       | Yes       | No         | Yes         | Yes         | Yes        |
| M279       | No        | No         | No          | No          | No         |

### Efficacy-dependent changes

| In chemical shifts | No  | —   | Yes | No  | No  | No  | No  | Yes | Yes |
|--------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| In signal intensities† | No  | —   | Yes | No  | No  | No  | No  | Yes | Yes |

*TM and ECL stand for transmembrane helix and extracellular loop, respectively.
†The signal intensities were normalized by dividing the observed intensities with the concentrations of β2-AR, estimated from the SDS-PAGE, and the numbers of scans.
‡Normalized chemical shift differences were < 0.1 p.p.m.
§In a complex manner, depending on both the populations and the exchange rates between the m82-β AR methionine resonances in the states with various efficacies.

Figure 3 | 1H-13C SOFAST-HMQC spectra of [methyl-13C-Met] β2-AR and their assignments. (a,b) 1H-13C SOFAST-HMQC spectra of [methyl-13C-Met] β2-AR in the carazolol-bound state (a) and in the formoterol-bound state (b). (c,d) 1H-13C SOFAST-HMQC spectra of the [methyl-13C-Met] β2-AR 4Met mutant in the carazolol-bound state (c) and in the formoterol-bound state (d). (e,f) 1H-13C SOFAST-HMQC spectra of the [methyl-13C-Met] β2-AR 4Met/M82V mutant in the carazolol-bound state (e) and in the formoterol-bound state (f). The regions with methionine chemical shifts are shown, and the assigned resonances are indicated. The resonances from M40 in the carazolol-bound state, and M40, M215 and M279 in the formoterol-bound state were not observed. Resonances indicated with single asterisks are derived from minor impurity proteins from insect cell membranes, but not from β2-AR. Double asterisks are from the intense DDm signal with an 1H chemical shift of 1.6-1.7 p.p.m.
The HMQC spectra of the β₂AR without the 4Met mutation were also recorded in the state bound with alprenolol, tulobuterol and clenbuterol, as well as carazolol and formoterol (Supplementary Fig. S11). As a result, the chemical shift changes observed for the β₂AR without the 4Met mutation were almost identical to those for the 4Met mutant, although the M82 resonances partially overlapped with the resonances from M96 or M98. Therefore, we can rule out the effect of the 4Met mutation on the efficacy in each ligand-bound state.

The differences in the other methionine resonances in each ligand-bound state are summarized in Fig. 6 and Table 1. The resonances from M36, M96/M98, M156 and M171 exhibited only small chemical shift differences in each ligand-bound state (Fig. 6 and Table 1).

**Discussion**

Different spectrum patterns from the M82^{2,33} methyl group are observed, depending on the efficacy of the bound ligand: the resonances of M82\(^{U}\) and M82\(^{D}\) in the inverse agonist-bound state, two resonances with slightly different chemical shifts from M82\(^{U}\) and M82\(^{D}\) in the neutral antagonist-bound state, one resonance with a chemical shift between M82\(^{U}\) and M82\(^{A}\) in the partial agonist-bound states and the resonance of M82\(^{A}\) in the full agonist-bound state (Fig. 4b–d).

Based on the comparison between the β₂AR crystal structures in the forms bound with an inverse agonist and with both a full agonist and a G-protein, the following structural mechanism for GPCR activation was proposed: full agonists induce a conformational change of S207\(^{5,46}\) and a subsequent rearrangement of the interactions between the TM helices in the middle of the TM region; the inward shifts of TM5 and TM7 at I121\(^{298}\) and N318, respectively, resulting in a large outward movement of the cytoplasmic half of TM6 (Supplementary Fig. S2). The M82^{2,33} side chain also exhibits a dramatic conformational change upon activation, and this conformational change strongly correlates with the rearrangement
**Figure 5** | The difference in $\beta_2$AR M2155-54 or M2796-41 resonances in the states with various efficacies. (a) $^{1}H^{13}C$ SOFAST-HMOC spectra of $[\alpha,\beta,\beta^{2}H_{3},\text{methyl}^{13}C\text{-Met}]\beta_2$AR/4Met at 298 K in the carazolol-bound (black), alprenolol-bound (cyan), tulobuterol-bound (green), and clenbuterol-bound (blue) and formoterol-bound (red) states. Only regions with M36 and M215 resonances are shown. (b) Overlay of the spectra shown in panel a, except for the formoterol-bound state, with the same colors. In panel b, only the regions with M215 resonances are shown. The centers of the resonances from M215 are indicated with dots. (c) Overlay of the regions with M279 resonances of the $^{1}H^{13}C$ SOFAST-HMOC spectra of $[\alpha,\beta,\beta^{2}H_{3},\text{methyl}^{13}C\text{-Met}]\beta_2$AR/4Met at 298 K in the carazolol-bound (black), alprenolol-bound (cyan), tulobuterol-bound (green) and clenbuterol-bound (violet) states. The centers of the resonances from M279 are indicated with dots. The line shapes of the M279 resonances are distorted due to the overlaps with the $t_1$ noises derived from the intense DDM signal with an $^{1}H$ chemical shift of 1.6–1.7 p.p.m.

![Diagram of receptor states](image_url)

**Figure 6** | Normalized chemical shift differences of the methionine methyl resonances between the carazolol- and formoterol-bound states. Normalized chemical shift differences, $\Delta\delta$, were calculated by the equation $\Delta\delta = (\Delta\delta_{1H}^{2} + \Delta\delta_{13C}^{2})^{1/2}$. The normalization factor (3.5) is the ratio of the s.d. of the methionine methyl $^{1}H$ and $^{13}C$ chemical shifts, deposited in the Biological Magnetic Resonance Data Bank (http://www.bmrb.wisc.edu/). The error values were calculated by the formula $\Delta\delta_{1H}^{2} + \Delta\delta_{13C}^{2}$. The number of replicates is greater than two. For M82, both of the $\Delta\delta$s between M82U and M82A (M82U/A), and M82D and M82A (M82D/A) were calculated. For M215 and M279, we could not calculate the $\Delta\delta$s between the carazolol- and formoterol-bound states, because these resonances were not observed in the formoterol-bound state, although $\Delta\delta$s between the carazolol- and clenbuterol-bound states, which should be smaller than those between the carazolol- and formoterol-bound states, were greater than 0.1 p.p.m.

![Normalized chemical shift difference graph](image_url)

of the interactions between I1213-40 and F2826-44 (Supplementary Fig. S3), via the inward shift of TM7 at Y3167,43 and S3197-46 and the axial shift of TM3 at C1163-35 (Supplementary Fig. S2). Therefore, the side-chain conformation of M822-53 would be sensitive to the activation state of $\beta_{2}$AR. On the other hand, M822-53 is 5–8 Å away from the conserved $\beta$-OH groups of the $\beta_{2}$AR ligands, and is not directly involved in the ligand binding. Therefore, the resonances from M822-53 would be less affected by the direct interactions with ligands. This is supported by further observation that the affinity of the antagonist [H]DHA for the 4Met/M82V mutant was almost identical to that for the 4Met mutant (Supplementary Fig. S7).

The $^{13}C$ and $^{1}H$ chemical shifts of the methionine methyl signals are reportedly affected by the side-chain conformation and the local environments, including the ring current effects from the neighbouring aromatic rings, respectively. Based upon the crystal structures of $\beta_{2}$AR, we propose that the M82U and M82D signals correspond to the inactive states that cannot directly activate G-proteins, and the M82A signal corresponds to the active state that can interact with G-proteins (Supplementary Discussion). Therefore, the differences between the conformations corresponding to M82U and M82D are found in a limited region close to the ligand-binding site. In contrast, the differences between the conformations corresponding to M82U/D and M82A are found in the TM region. Hereafter, we refer to these conformations corresponding to M82U, M82D and M82A as the M82U conformation, the M82D conformation and the M82A conformation, respectively.

Previous comprehensive analyses of efficacies in various ligands revealed that the efficacies increase in the following order: an inverse agonist, carazolol; a neutral antagonist, oxprenolol, which is an analogue of alprenolol; a weak partial agonist tulobuterol; a partial agonist, clenbuterol; and a full agonist, formoterol (Supplementary Table S1). In the neutral antagonist-bound and partial agonist-bound states, the M822-53 resonances exhibited chemical shifts between M82U and M82A in an efficacy-dependent manner (Fig. 4c). The resonances from M822-53 in the partial agonist-bound states shifted towards M82A as the temperature was lowered from 298 K to 283 K (Supplementary Fig. S8a,b). The temperature-dependent shifts, together with the chemical shifts in...
an efficacy-dependent manner, suggest that β2AR exists in equilibrium between the M82D and M82A conformations in the partial agonist-bound states, with faster exchange rates than the chemical shift difference. The observation of two resonances from M82D, in the carazolol- and alprenolol-bound states suggests that β2AR exists in equilibrium between the M82D and M82A conformations in the inverse agonist- and neutral antagonist-bound states, with slower exchange rates than the chemical shift difference. The two resonances from M82D in the alprenolol-bound state were closer to M82D than those in the carazolol-bound state, suggesting that the minor population of the M82A conformation, in equilibrium with the M82D conformation, would also exist in this state, as supported by the simulation of M82D resonances (Supplementary Discussion). The incomplete linearity of the efficacy-dependent chemical shift change might be derived from another equilibrium within the M82A conformation.

Similar phenomena were also observed for the M215 and M279 resonances. The M215 and M279 resonances exhibited the chemical shifts and intensities in an efficacy-dependent manner (Fig. 5 and Table 1), and these resonances shifted towards those in the carazolol-bound state at lower temperature (Supplementary Fig. S10a and b). The simulations of these resonances, with the same parameters as that of the M82 resonances, were in good agreement with the observed spectra (Supplementary Discussion). Therefore, the efficacy-dependent conformational equilibrium between the M82D, and M82A conformations observed for M82D accompanies large conformational changes on TM5 and TM6.

Based on the above structural interpretation of the resonances from M82, we propose the following signal regulation mechanism. In the full agonist formoterol-bound state, most of the β2AR molecules assume the active conformation (Fig. 7a). In the partial agonist tulobuterol- and clenbuterol-bound states, β2AR exists in an equilibrium between the inactive and active conformations (Fig. 7b), and the populations of the two conformations determine the efficacies. This is in good agreement with the significant, but reduced, efficacy for tulobuterol and clenbuterol2,4. In the neutral antagonist alprenolol-bound state, β2AR exists in equilibrium between two major inactive conformations, which are different only in the region close to the ligand-binding site, and one minor active conformation (Fig. 7c). The weak basal activity is due to the existence of the minor M82A conformation. In the inverse agonist-bound state, β2AR exists in equilibrium between the two locally different inactive conformations (Fig. 7d). Considering that the basal activity partly remained even in the carazolol-bound state16,24, there may be a minor population of the M82A conformation in this state, in an equilibrium with one of the inactive conformations.

Two mechanisms have been proposed for the partial efficacies in the partial agonist-bound states: the equilibrium between the active and inactive states, and the distinct conformation of the partial agonist-bound state from those of both the active and inactive states3, although there have been no experimental evidence for them. The chemical shifts of the M82D,5,3 resonances in the efficacy-dependent and the temperature-dependent manner in the partial agonist-bound states suggest the equilibrium between the active and inactive states.

The M82D resonance was observed only in the inverse agonist- and neutral antagonist-bound states, and the intensity of the M82D resonance is higher in the inverse agonist-bound state than in the neutral antagonist state. In addition, the 13C chemical shifts of M82U and M82D suggest that the M82U conformation flexibly adopts both the trans and gauche conformations, whereas the M82D conformation mainly adopts the trans conformation (Supplementary Discussion); therefore, the M82D conformation may be more rigid than the M82U conformation. It is thus tempting to speculate that the M82D conformation is more refractory to shifting to the activated state than the M82U conformation, and the higher population of the M82D conformation in the inverse agonist-bound state causes the inhibition of the basal activity.

The resonances from M215 and M279, which are located on the cytoplasmic side of the TM region, were not observed in the formoterol-bound state, suggesting that these signals were broadened due to the conformational exchange between the multiple

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**Figure 7** A proposed mechanism for the differences in the efficacy of β2AR for different ligands. β2AR adopts three conformations with different M82D environments; the M82A conformation induces signalling, whereas the M82U and M82D conformations do not. M82U and M82A conformations are largely different on TM5 and TM6, whereas the differences between M82D and M82U conformations are localized in the region close to the ligand-binding site. (a) In the full agonist formoterol-bound state, β2AR adopts mostly the M82A conformation, exhibiting almost full efficacy of β2AR. (b) In the partial agonist clenbuterol- and tulobuterol-bound states, β2AR exists in equilibrium between the M82A and M82U conformations, exhibiting the significant signaling with reduced efficacies. In the tulobuterol-bound state, where the efficacy is lower than that of the clenbuterol-bound state, the populations of the M82U conformation are larger. (c) In the neutral antagonist alprenolol-bound state, β2AR adopts mostly the M82A and M82D conformations, in equilibrium with a small population with the M82U conformation. The presence of the small population with the M82A conformation accounts for the basal activity of β2AR. (d) In the inverse agonist carazolol-bound state, β2AR exists in equilibrium between the M82D and M82C conformations, exhibiting the inhibition of basal activities described in panel c.
conformations in the formoterol-bound state. The multiple conformations on the cytoplasmic side of the TM region in the full agonist-bound state may be effective for interacting with various effectors in the conformational selection manners29,30. A number of β2AR ligands have been shown to impart differing degrees of signalling in G-protein and arrestin pathways, a phenomenon called ‘functional selectivity’ or ‘biased signalling’20,28–32. The biased signalling is important for both understanding the functions ofGPCRs and drug development, and it is quite interesting to investigate the conformations of β2AR in the biased ligand-bound states. Although biased signalling was not reported for the ligands chosen in this study30, the biased signalling may be due to the diversity of the conformational dynamics in the cytoplasmic side of the TM region in each ligand-bound state.

The conformational differences between the crystal structures of β2AR were also basically observed in those of adenosine A2A receptor and rhodopsin33–35. In addition, methionine residues are highly abundant in TM3, TM5 and TM6, as described above (Supplementary Fig. S1), and can be observed without any chemical modification. Therefore, the methionine 1H-13C resonances, including Met263–28, are broadly applicable for the analyses of the bound ligand efficacy-dependent conformational differences in various GPCRs.

**Methods**

**Reagents and buffers.** All reagents were from Nacalai Tesque, unless otherwise noted. The (αβββ-H3)Met1-13C methylome was prepared by the enzymatic deamination of [methyl-13C]methionine (ISOTEC or Cambridge Isotope Laboratories) with Escherichia coli cystathionine-γ-synthase, as previously described36. Alprenolol-cysteine (AlpC) was synthesized as previously described37. Affi-gel/AIpC was prepared by coupling 5 mg ml−1 AlpC solution with Affi-gel10 (Bio-Rad) in a dimethyl sulfoxide (DMSO, Dojindo) solution, according to the manufacturer’s instructions. Carazolol (Wako Chemicals) and formoterol (Sigma) were solubilized at 100 mM in DMSO-d6 (ISOTEC), and diluted with buffers. Tulobuterol (Wako Chemicals) was solubilized at 400 mM in methanol-d4 (ISOTEC), and diluted with buffers. Alprenolol (Sigma) and clenbuterol (Wako Chemicals) were solubilized at 100 mM in D2O (Cambridge Isotope Laboratories), and diluted with buffers.

**Generation of β2AR recombinant baculovirus.** The complementary DNA fragment encoding human β2AR (Gly2-Gly365), with an N-terminal FLAG-tag (DYKDDDDA) and a C-terminal decahistidine-tag was amplified by PCR and cloned into the pFastBac1 vector (Invitrogen) via the BamHII-Xbal sites. The sequence encoding the gp64 signal sequence was amplified from the pBAC-6 vector (Novagen), and placed immediately 5′ to the FLAG-tag. Mutations were introduced by either a QuikChange site-directed mutagenesis kit or a QuikChange multi site-directed mutagenesis kit (Stratagene). E122W, N187E and C265A mutants were obtained per 0.8 l of insect cell culture.

**Expression and purification of β2AR.** The expressSF + cells (SF + cells, Protein Sciences) were routinely maintained at 27 °C in 100 ml SF-900 II serum-free medium (GIBCO), with 50 international units per ml penicillin, 50 µg ml−1 streptomycin and 0.125 mg ml−1 amphotericin B. Recombinant baculoviruses were generated and amplified with the Bac-to-Bac system (Invitrogen), according to the manufacturer’s instructions.

**Expression and purification of β2AR.** The expressSF + cells (SF + cells, Protein Sciences) were routinely maintained at 27°C in 100 ml SF-900 II serum-free medium (GIBCO), with 50 international units per ml penicillin, 50 µg ml−1 streptomycin and 0.125 mg ml−1 amphotericin B, in a 250 ml Erlenmeyer flask (Corning) on an orbital shaker (130 rpm). For the expression of [methyl-13C-Met]β2AR, SF + cells in SF-900 II medium were centrifuged at 200g, and resuspended in methionine-depleted ESF921 medium (Expression Systems), at about 2×109 cells per ml. For the expression of non-labeled β2AR, SF-900 II medium was supplemented with 1% w/v dodecyl-β-D-maltoside (DDM, Dojindo) for 4 h, and then centrifuged at 100,000×g for 30 min. The supernatant was batch incubated overnight with 1 ml of TALON metal affinity resin (Clontech). The resin was washed with 30 ml of buffer B, supplemented with 0.1% DDM and 20 mM imidazole. The protein was eluted with 8 ml of buffer B, supplemented with 0.1% DDM and 200 mM imidazole.

For the ligand-affinity chromatography, the eluate from the TALON affinity resin was batch incubated for 4 h with 0.5 ml of Affi-gel/AlpC. The resin was washed with 10 ml of buffer B, and supplemented with 0.1% DDM and 1 mM formoterol. For the concentration and the ligand exchange of β2AR, a second TALON affinity step was performed after the ligand-affinity chromatography. The eluate from the ligand-affinity chromatography was batch incubated for 1 h with 0.5 ml of TALON resin. The resin was washed with 5 ml of buffer B, supplemented with 0.1% DDM and 100 µM formoterol. The ligand exchange from formoterol to clenbuterol or tulobuterol was accomplished by further washing of the resin for about 3 h, with 25 ml of buffer B, supplemented with 0.1% DDM and 1 mM ligands (clenbuterol or tulobuterol). The protein was eluted with 1 ml of buffer B, supplemented with 0.08% DDM, 200 mM imidazole and the ligands (100 µM formoterol, 1 mM clenbuterol or 1 mM tulobuterol).

The eluate from the second TALON affinity step was concentrated using a centrifugal filter device (Amicon Ultral-4, 30 kDa molecular weight cutoff, Millipore), with Sephacryl S200 column equilibrated with buffer C (20 mM sodium phosphate, pH 7.1, H2O/D2O = 1/99), supplemented with the ligands (100 µM formoterol, 1 mM clenbuterol or 1 mM tulobuterol). In total, 50–400 µg of β2AR and its mutants were obtained per 0.81 insect cell culture.

**NMR experiments.** All of the spectra were recorded with a Bruker Avance 800 spectrometer equipped with a cryogenic probe, and were processed by Topspin 2.1 (Bruker).

The assignment of the methionine methyl 1H-13C resonances of β2AR was accomplished by the following procedure. 1H-13C SOFAST-HMQC spectra2 and 1H-13C HMQC spectra with echo/anti-echo gradient coherence selections38 were recorded for 5–40 µM [methyl-13C-Met]β2AR and its mutants in buffer C, supplemented with 100µM formoterol at 298 K. After the NMR experiments, carazolol was added to a final concentration of 500 µM, and 1H-13C SOFAST-HMQC spectra and 1H-13C HMQC spectra with echo/anti-echo gradient coherence selections were recorded at 298 K, for the assignment of the methionine methyl 1H-13C resonances in the carazolol-bound states. 1H-13C HMQC spectra with echo/anti-echo gradient coherence selections were utilized to discriminate the methionine resonances from the t9 noises derived from the intense DDM signals in the 1H-13C SOFAST-HMQC spectra.

The structures of β2AR in the various ligand-bound states were investigated by the following procedure. 1H-13C SOFAST-HMQC spectra were recorded for 20 µg [methyl-13C-Met]β2AR and its mutants in buffer C (20 mM sodium phosphate, pH 7.1, H2O/D2O = 1/99), supplemented with the ligands (100 µM formoterol, 1 mM clenbuterol or 1 mM tulobuterol). For the NMR experiments in the formoterol- and clenbuterol-bound states, carazolol and alprenolol were added to final concentrations of 500µM and 1 mM, respectively, and 1H-13C SOFAST-HMQC spectra were recorded at 283 K and 298 K.

1H-13C SOFAST-HMQC spectra were recorded by excitation with a 4-ms PC9 120 degree pulse29 and the inversion of a 2-ms Q3 180 degree pulse40. For all of the spectra, the spectral widths were set to 12,800 Hz and 4,800 Hz for the 1H and 13C dimensions, respectively, and the inter-scan delays were set to 1 s. For the spectra recorded at 298 K in Supplementary Fig S5a–h, 1,024×128 complex points were zero-filled to 256×256 and 236 scans/256 complex points were recorded and 128 scans/FID gave rise to an acquisition time of 10 h for each spectrum. For the other spectra recorded at 298 K, 1,024×256 complex points were recorded, and 128 scans/FID gave rise to an acquisition time of 15 h for each spectrum. All the spectra were referenced with 3-(trimethylsilyl)-1-propanesulfonylic acid sodium salt in both 1H and 13C dimensions.

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