Analysis of $\beta_2$AR-Gs and $\beta_2$AR-Gi complex formation by NMR spectroscopy

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The $\beta_2$-adrenergic receptor ($\beta_2$AR) is a prototypical G protein-coupled receptor (GPCR) that preferentially couples to the stimulatory G protein Gs and stimulates CAMP formation. Functional studies have shown that the $\beta_2$AR couples to inhibitory G protein Gs, activation of which inhibits cAMP formation [R. P. Xiao, Sci. STKE 2001, re15 (2001)]. A crystal structure of the $\beta_2$AR-Gs complex revealed the interaction interface of $\beta_2$AR-Gs and structural changes upon complex formation [S. G. Rasmussen et al., Nature 477, 549 (2011)], yet, the dynamic process of the $\beta_2$AR signaling through Gs and its preferential coupling to Gs over Gi is still not fully understood. Here, we utilize solution nuclear magnetic resonance (NMR) spectroscopy and supporting molecular dynamics (MD) simulations to monitor the conformational changes in the G protein coupling interface of the $\beta_2$AR in response to the full agonist BI-167107 and Gs and Gi. These results show that BI-167107 stabilizes conformational changes in four transmembrane segments (TM4, TM5, TM6, and TM7) prior to coupling to a G protein, and that the agonist-bound receptor conformation is different from the G protein coupled state. While most of the conformational changes observed in the $\beta_2$AR are qualitatively the same for Gs and Gi, we detected distinct differences between the $\beta_2$AR-Gs and the $\beta_2$AR-Gi complex in intracellular loop 2 (ICL2). Interactions with ICL2 are essential for activation of Gi. These differences between the $\beta_2$AR-Gs and $\beta_2$AR-Gi complexes in ICL2 may be key determinants for G protein coupling selectivity.

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known about the conformational dynamics of the β2AR in response to different G protein subtypes. In this study, we utilized 13C-dimethylated lysine as an NMR probe to investigate the conformational changes of several sites in the intracellular G protein binding interface of the β2AR. 13C-dimethylated lysine is an ideal probe for this purpose because of its large, flexible side chain, enabling studies of larger protein complexes. Our studies show that in addition to TM6, agonist binding can also promote

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**Fig. 1.** The β2AR-G protein signaling pathways. (A) The agonist-bound β2AR activates either Gs or Gi heterotrimer, which stimulates or inhibits the adenylyl cyclase activity, respectively. (B) Structure of β2AR-Gs complex (PDB ID code: 3SN6), the lysine residues that are chosen as NMR probes are shown as solid spheres, other lysine residues were mutated to arginine as described in the text. (C–E) The lysine probes undergo conformational changes during the activation of the β2AR, as shown by cytoplasmic views of active β2AR (PDB ID code: 3SN6) (C), inactive β2AR (PDB ID code: 2RH1) (D), and the overlap of active and inactive β2AR (E).
conformational changes in TM4, TM5, and TM7. We observe further structural changes in β2AR coupled to Gs and G1i. While the structural changes stabilized by Gs and G1i are qualitatively similar in most cytoplasmic domains probed, we detected distinct differences between the β2AR-Gs and the β2AR-G1i complex in intracellular loop 2 (ICL2). In the inactive-state structure of the β2AR, ICL2 is a loop, while in the β2AR-Gi complex, ICL2 forms an α-helix that positions F13934.51 in ICL2 to engage a hydrophobic pocket in Gαi, thereby triggering nucleotide release (4).

Results
Design of Modified βARs for 13C-Dimethylated Lysine NMR Studies.
13C-dimethylated lysine probes have long served as excellent probes for NMR experiments due to high sensitivity and relatively long transverse relaxation times (18). The WT β2AR contains 16 lysine residues, most of which are located at the G protein coupling interface (Fig. 1 B–E and SI Appendix, Fig. S1A). Therefore, these lysines can be used as NMR reporters to monitor structural changes in response to agonists and G proteins.

However, previous studies have shown that the 13C-dimethylated lysine spectra of C-terminally truncated WT β2AR (β2AR-365N) was too crowded to make assignments (11), probably due to the similar chemical environments of these intracellular lysines. To avoid α-helix TR signals overlapping, we made a truncated version of the WT β2AR construct (β2AR-365N-K601E) by mutating all lysines to arginines as previously reported (19) and removing the C-terminal 48 residues (SI Appendix, Fig. S1 A and D). Radio-ligand binding studies show that these modifications have only small effects on the affinity of antagonists and agonists (SI Appendix, Fig. S1C). A previous study showed that the lysine-free β2AR has functional properties that are comparable to WT β2AR in stimulation of adenyl cyclase activity (19) and the recruitment of β-arrestin (20). The only reported effect of removing lysine is loss of ubiquitination and subsequent targeting receptor to lysosome for degradation (21). We then individually introduced eight lysines to different transmembrane segments in the intracellular domain of the β2AR based on the lysine-free construct (Fig. 1 B–E and SI Appendix, Fig. S1A). These eight residues were K601.59, K140.32, K147.40, K227.29, K267.29, K273.35, K328K7.55, and R333K8.51 (superscripts are Ballesteros and Weinstein numbering; ref. 21), located at the intracellular end of TM1, TM4, TM5, TM6, TM7, ICL2, TM4, and H8. The resulting eight constructs were expressed, purified, and labeled before through reductive methylation (SI Appendix, Figs. S1B and S2A). Methylation efficiency was high, as determined by loss of labeling with fluorescamine, an amine reactive fluorophore (SI Appendix, Fig. S1B). We applied 1H,13C heteronuclear single quantum coherence spectroscopy (1H-13C HSQC) to each construct. The signal of each lysine was observed in the 1H-13C dimension as compared to the spectrum of lysine-free β2AR (SI Appendix, Fig. S3 A–H). The signals are unlikely from the N terminus because the N-terminal Flag tag was removed after methylation. In order to make sure they are not residual N terminus signals due to incomplete protein digestion, we measured the signals from the methylated N terminus and they are in different positions from the lysine signals (SI Appendix, Fig. S3). For each β2AR-365N-one-K construct, we obtained the 1H-13C HSQC spectra under the following conditions: unliganded (apo-state) β2AR, BI-167107-bound, carazolol-bound, BI-167107-bound β2AR in complex with Gs or G1i in the presence of 300 μM GDP (G GDP or G1i GDP), and BI-167107-bound β2AR in complex with nucleotide-free Gs or G1i (G EMPT Empty or G1i EMPT Empty). For these studies we used saturating concentrations of BI-167107, a potent full agonist with a dissociation half-life of 400 min and an association half-life of less than 4.4 min (12). For inverse agonist, we used a saturating concentration of BI-167107, F, and H8. The resulting eight constructs were expressed, purified, and labeled before through reductive methylation (SI Appendix, Figs. S1B and S2A). Methylation efficiency was high, as determined by loss of labeling with fluorescamine, an amine reactive fluorophore (SI Appendix, Fig. S1B). We applied 1H,13C heteronuclear single quantum coherence spectroscopy (1H-13C HSQC) to each construct. The signal of each lysine was observed in the 1H-13C dimension as compared to the spectrum of lysine-free β2AR (SI Appendix, Fig. S3 A–H). The signals are unlikely from the N terminus because the N-terminal Flag tag was removed after methylation. In order to make sure they are not residual N terminus signals due to incomplete protein digestion, we measured the signals from the methylated N terminus and they are in different positions from the lysine signals (SI Appendix, Fig. S3). For each β2AR-365N-one-K construct, we obtained the 1H-13C HSQC spectra under the following conditions: unliganded (apo-state) β2AR, BI-167107-bound, carazolol-bound, BI-167107-bound β2AR in complex with Gs or G1i in the presence of 300 μM GDP (G GDP or G1i GDP), and BI-167107-bound β2AR in complex with nucleotide-free Gs or G1i (G EMPT Empty or G1i EMPT Empty). For these studies we used saturating concentrations of BI-167107, a potent full agonist with a dissociation half-life of 400 min and an association half-life of less than 4.4 min (12). For inverse agonist, we used a saturating concentration of carazolol. The very slow dissociation half-lives for these ligands ensures that they are bound throughout the NMR experiment.

There are crystal structures of carazolol-bound β2AR (22) and the BI-167107-bound β2AR-Gi complex (4); however, it has not been possible to crystalize native β2AR in the apo-state or bound to agonist alone, most likely due to the inherent instability of the receptor under these conditions. While β2AR also couples to Gs, there are no reported structures of a β2AR-Gs complex. Therefore, the NMR experiments provide structural insights into the apo-state, the agonist-bound β2AR, and the β2AR-Gi complex relative to available crystal structures. Figs. 2–4 show the comparison of 1H-13C HSQC spectra of the eight 13C-dimethylated lysine probes in TM1, ICL2, TM4, TM5, TM6, TM7, and Helix-8 in four different conditions: apo-state, inverse agonist-bound, agonist-bound, and agonist + Gs EMPT Empty.

Apo-β2AR Compared to Inverse Agonist-Bound States. The β2AR exhibits basal activity for Gs activation in the apo-state. This activity can be suppressed by inverse agonists such as carazolol. Previous 13C-methionine and 19F NMR studies revealed relatively subtle conformational changes when comparing inverse-agonist stabilized inactive-state and the apo-state β2AR (12, 13). Consistent with these findings, our results show relatively small spectral changes in several domains of the intracellular surface in apo-state and the carazolol-stabilized inactive state. We observe the largest difference between the apo-state and carazolol-bound inactive state is in K601.59 (Fig. 2A), suggesting a change in its chemical environment possibly due to a change in polar interactions with the end of Helix 8 (Fig. 2 C–F). In the apo-state, K601.59 is represented by strong Peak 1 with a weak shoulder Peak 2. In carazolol-bound receptor we observe an up-field shift in Peak 1 and no change in Peak 2.

In the apo-state we observe two peaks for K1474.39 at the end of TM4 (Fig. 3). K227.35 in TM6s, and R332K7.55 in TM7 (Fig. 4). This suggests the existence of conformational heterogeneity in these receptor domains with exchange between two states on slow time scale (seconds or slower). In K273.35 and R328K7.55 we observe very small up-field shifts in the position of Peak 1 in carazolol-bound receptor compared to the apo-state that are in the opposite direction observed for agonist (Fig. 4 C and D).

Conformational Changes following Agonist Binding and Gs Coupling.
To correlate structural changes observed by NMR with the β2AR-Gs crystal structure, we obtained spectra of the β2AR bound to nucleotide-free Gs (β2AR-Gs EMPT Empty), β2AR-Gi EMPT Empty was formed by adding the nucleotidase apyrase after complex formation to degrade any released GDP. In the β2AR-Gs EMPT Empty crystal structure, the largest structural changes are the outward movement of TM5 and TM6 and inward movement of TM7, which allows the insertion of the α5 helix of Gαs (Fig. 1 C–E and SI Appendix, Fig. S4). We observed obvious spectral changes when comparing β2AR bound to agonist alone with the β2AR-Gs EMPT Empty complex in all probes except K333K8.51 in Helix 8 (Figs. 2–4). This may be due to a combined effect of direct Gs protein binding, as well as a fully active conformation stabilized only by agonist and Gs protein, but not agonist alone (13).

ICL1 and helix 8. The spectrum of K601.59 at the end of TM1 is nearly identical for apo-state and agonist-bound state (Fig. 2A), suggesting little perturbation of conformational dynamics in the junction of TM1 and ICL1. ICL1 and Helix 8 have no direct interaction with Gs in the β2AR-Gs EMPT Empty complex. The observation of intensity reduction upon coupling with Gs for K601.59 (Fig. 2A) and K333K8.51 (Fig. 2B) may result from the slower tumbling due to the formation of complex with a much larger molecular weight. We do observe a change in the spectra of K601.59 upon coupling to Gs. In the inactive state K601.59 forms a polar interaction with E338K8.50 in Helix 8 (Fig. 2 E and F). This is lost in the β2AR-Gi EMPT Empty complex due to a small ~1.5 Å movement of...
TM1 away from Helix 8 (Fig. 2 C and D). We also observe the appearance of a weak downfield peak in R333K8.51 in H8.

ICL2. In the inactive state structure of the β2AR, ICL2 has no secondary structure while it is an alpha-helix in the β2AR-Gs complex (Fig. 3 C and D). The helix positions F334.33 in ICL2 to engage a hydrophobic pocket formed by H41, V217, F376, C379, R380, and E383 in the Gαs subunit (Fig. 3D). This interaction is essential for Gs activation (4, 23, 24). The conformational changes detected in the NMR spectra of K147.39 and K140.52 may represent the chemical environment change during the transition of ICL2 from a loop to a helix (Fig. 3 A and B).

K147.39 is located at the junction of TM4 and ICL2 (Fig. 1 C and D). In the inactive state, K147.39 forms a hydrogen bond with the backbone carbonyl of O65.12.51 in ICL1 (Fig. 3E). This interaction is lost in the structure of β2AR-Gs empty (Fig. 3F). In the apo state, the two peaks likely represent an equilibrium between the formation (Peak 2) and disruption (Peak 1) of the hydrogen bond in slow exchange (Fig. 3B). The observation of one dominant peak for K147.39 in the agonist-bound state compared to the two peaks in the apo-state most likely suggests faster exchange rate between the different conformations observed in the apo state (Fig. 3B). In β2AR-Gs complex we observe predominantly Peak 1, consistent with the loss of the hydrogen bond (Fig. 3B).

In contrast to the dynamics in the junction of TM4 and ICL2 observed in the apo and agonist-bound receptor, we observe a strong single peak for K140.52 at the junction of ICL2 and TM5 (Fig. 3A). In the inactive state, K140.52 forms a hydrogen bond with Q229.58 in TM5, while in the β2AR-Gs complex it is adjacent to the β3AR-Gs interface (Fig. 3 C and D). Coupling with Gs dramatically alters the chemical environment of K140.52, as evidenced by the appearance of a new downfield peak (labeled as peak2). Only a small fraction of Peak 1 remains following the formation of β2AR-Gs empty, consistent with a minor population of uncoupled β2AR (Fig. 3A).

TM5, 6, and 7. While we observe spectral changes in K227.56, K267.29, K273.35, and K328.72 upon agonist binding, further changes are observed following G protein coupling (Fig. 4 A–D), consistent with changes observed in TM5, TM6, and TM7 in the β2AR-Gs structure (Fig. 4 E and F). The slight broadening of the peak representing K227.56 upon agonist binding suggests the appearance of two or more conformations in intermediate exchange at the intracellular side of TM5 (Fig. 4A). Upon coupling to Gs, we see the appearance of a new downfield peak (Peak 2) and a weaker Peak 1 (Fig. 4A).

The two TM6 probes, K267.29 and K273.35, show significant differences in terms of peak patterns. In the apo and agonist-bound state K273.35 is represented by two weak peaks, indicating that there are at least two conformations with a slow exchange rate, while K267.29 is represented by a stronger single peak (Fig. 4 B and C). This is because K267.29 is located at the end of TM6 and its side chain is expected to exhibit relatively high conformational flexibility, whereas K273.35 is located in the transmembrane core and its side chain can interact with neighboring residues I277.39 and R328.72 (Fig. 4 E and F). Upon agonist binding, there is a downfield shift in the peak representing...
There are two peaks for K2736.35 in the β2AR-Gs complex (Fig. 4B). Peak 2 has the same chemical shift seen with agonist binding or G protein coupling preferences. As noted above, the β2AR can couple to both Gs and Gi. However, efforts toward obtaining the structure of β2AR-Gs complex have failed due to the relatively weak interaction and instability of the complex. As a consequence, little is known about differences in the interactions between β2AR and Gi compared with the β2AR-Gs complex.

To provide more structural insights into the basis of β2AR coupling selectivity for Gi over Gs, we next sought to compare β2AR binding with Gi and Gs by using the 1H-13C HSQC spectra of the β2AR-BI-167017-Gi

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complex (Fig. 5). The β2AR-Gi
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complex was formed by adding the nucleotide-ase apyrase after complex formation to degrade any released GDP. Fig. 5 compares the spectra of β2AR-Gs
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and β2AR-Gi
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complexes. Consistent with the previous observation that the β2AR-Gi
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complex is less stable than the β2AR-Gs complex (29), we observed smaller spectral changes in the β2AR-Gi
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complex sample. The NMR spectral changes of K2736.35, K2676.29, K2736.35 and R328K7.55 corresponding to the conformational changes of TM5, 6, and 7 are similar to those obtained from the β2AR-Gs complex; however, peak 2 of K2275.56 and K2676.29 may reflect different TM5–TM6 interactions in a small population of nucleotide-free β2AR-Gs complex.

It is interesting to note that the spectra of K2275.56, K2736.35 and R328K7.55 are similar (Fig. 4 C and D). This may be due to the close proximity of K2736.35 and R328K7.55 observed in the inactive state crystal structures (Figs. 1 C and D and 4E) where R328K7.55 could form a hydrogen bond with the backbone carbonyl of K2736.35. This interaction would be lost upon coupling with Gi. For both K2736.35 and R328K7.55, agonist binding leads to a downfield shift in Peak 1, and Peak 1 is lost in the β2AR-Gs complex. Peak 2 may represent a conformation that is more exposed to the solvent, therefore Peak 2 is less affected by agonist-binding or G protein coupling than Peak 1.

Conformational Differences in Gi and G11, Coupling. Recently, a number of GPCR-Gi
null

complex structures have been determined by cryo-EM (5–7, 25–28). The Gi and β2AR interaction interface is composed by TM3, TM5, TM6, and ICL2. While Gi
null

interactions with TM3, TM5, and TM6 are similar in GPCR-Gi
null

protein complexes, TM2, TM7, and H8 are also involved in formation of the A1 adenosine receptor (A1R)-Gi complex, and ICL2 and the junction between TM7 and H8 contribute to formation of the rhodopsin-Gi complex (SI Appendix, Fig. S5). For GPCR-Gi
null

complexes, the outward displacement of TM6 is smaller than observed for the β2AR-Gs complex. The differences in the interaction interface for these complexes might contribute to G protein coupling preferences. As noted above, the β2AR can couple to both Gs and Gi. However, efforts toward obtaining the structure of β2AR-Gs complex have failed due to the relatively weak interaction and instability of the complex. As a consequence, little is known about differences in the interactions between β2AR and Gi compared with the β2AR-Gs complex.

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Fig. 3. Conformational changes of ICL2 from inactive to active state. (A and B) 1H-13C HSQC spectra in H dimension are shown at the bottom. The local environment of the two probes are shown by side views in inactive (gray, C) and active (green, D, F) conformations. Gi
null

is shown in purple (D). The agonist BI-167107 is abbreviated by BI.
Fig. 4. Conformational changes of TM5, TM6, and TM7 during \(\beta_2\)AR activation. (A–D) The \(^1\)H-\(^{13}\)C HSQC spectra of K227\(^5,\)\(^7,\)\(^1\) (A), K267\(^6,\)\(^2\) (B), K273\(^6,\)\(^5\) (C), and K328\(^7,\)\(^5\) (D). The 1D slices of corresponding \(^1\)H-\(^{13}\)C HSQC spectra in H dimension are shown at the bottom. The local environments of the four probes are shown in inactive (gray, E) and active (green, F) conformations. The \(\alpha5\) helix from Gs\(\alpha\) is shown as purple cartoon. The agonist BI-167107 is abbreviated by BI.
Fig. 5. Comparison of conformational changes in β2AR coupled to Gs or Gi1. Bi is short for BI-167107. (A–H) The $^1$H-$^1$C HSQC spectra of the eight probes from TM1 to H8. The eight probes are K60 (A), K140 (B), K147 (C), K227 (D), K267 (E), K328 (F), K333 (G), and K333 (H). For each probe, the overlay of the $^1$H-$^1$C HSQC spectra of agonist-bound β2AR (green) and β2AR-Gi1 (blue) are shown at the top, followed by the comparison of the $^1$H-$^1$C HSQC spectra of agonist-bound β2AR (green) and β2AR-Gs (pink), then by the overlay of β2AR-Gs (blue) and β2AR-Gi1 (pink). The 1D slices of corresponding $^1$H-$^1$C HSQC spectra in H dimension are shown at the bottom.
due to the increase in mass. The lack of spectral changes for K1400.3.52 and K1471.3.52 suggest that ICL2 does not form an alpha helix when coupled to G11 and may have only weak interactions with G11, possibly explaining the less efficient coupling and the overall instability of the β2AR-G11 complex.

As noted above, the insertion of F13934.51 in ICL2 of the β2AR into a hydrophobic pocket formed by H41, V217, F376, C379, and R380 in the Gαs is essential for GDP release (SI Appendix, Figs. S6A and S7A). Of these, only F376 is conserved as F336 in G1 (SI Appendix, Fig. S6B). When comparing sequences of ICL2 from GPCRs that couple to Gαs, G11, Gq11, and G12, position 34.51 is most often a L, F, I, or M for G1 coupled receptors, while a broader range of amino acids are found in Gαs coupled receptors (SI Appendix, Fig. S6D). It is possible that interactions with 34.51 in ICL2 may be less important for coupling with G11 than G1. This is in agreement with the recent structures of G1 in complex with the cannabinoid receptor subtype 1 (CB1) (27), the μ-opioid receptor (μOR) (6), and the A1R (7). For these receptors, the interactions between the amino acid at position 34.51 in ICL2 (L in CB1 and A1R, and V in μOR) and G1 are much weaker, interacting with only one or two side chains of G1 (SI Appendix, Fig. S7B–D). However, this weaker interaction between ICL2 and G1 is not universal. For the neurotensin receptor subtype 1 (NTSR1), which couples promiscuously to G11, Gαs, and Gq11 (30), F17434.51 in ICL2 packs into a pocket formed by four amino acids in G1 (SI Appendix, Fig. S7E). The importance of the amino

![Fig. 6. Comparison of conformational changes measured by MD simulations in β2AR coupled to G1 or Gs. Backbone rmsd in Å extracted from 3 x 3-μs-long MD simulations of β2AR-G1 (pink) and β2AR-Gs (cyan) of structural elements at the receptor-G protein interface. (A) ICL1 of β2AR: E6212.48-Q6512.51. (B) ICL2 of β2AR: I1353.54-A1504.42. (C) H8 of β2AR: S3298.47-C3418.59. (D) α5 helix of Gαi and Gαs. (E) TM5 of β2AR: N1965.35-R239ICL3. (F) TM6 of β2AR: C2656.27-Q2996.61. The rmsd values were calculated relative to the respective structural element in the starting structure of β2AR-G1EMPTY and β2AR-GsEMPTY.](image)
acid in position 34.51 was initially demonstrated for the G_{d4}1 coupled M1 muscarinic receptor (MIR) where the substitution of L131^{4.51} for Ala in ICL2 led to a loss of G_{d4}1 coupling (24). In the recent cryo-EM structure of the MIR-G11 complex, L131^{4.51} packs into a pocket formed by four amino acids in G11 (28) (SI Appendix, Fig. S7F). Like G_{i}, coupledGPCRs, L, F, I, or M are more commonly found at position 34.51 in G_{d4}1 coupled GPCRs (SI Appendix, Fig. S6D). While the evidence suggests that weak interactions between F139^{34.51} in ICL2 of the β2AR and G_{i} may account for poor coupling efficiency, we cannot exclude the possibility that β2AR simply couples less efficiently to G_{i} and the lack of strong engagement with ICL2 is a manifestation of that.

Similar to G_{i}, G_{i1} binding also has little effect on R333K8.51 located at H8 (Fig. S5H). The decrease in intensity is likely due to the increased size of the complex. Notably, the decrease in intensity is greater for G_{i1-EMPTY} compared to G_{i1}, consistent with the formation of a more stable complex. The low intensity downfield peak observed in R333K8.51 with G_{i1} is not observed with the formation of a more stable complex. The low intensity downfield peak observed in R333K8.51 with G_{i1}. The spectra of K601.59 in TM1 are similar for G_{i1} and G_{i1}. The spectra of K601.59 in TM1 and K3287.55 in TM7; however, additional changes are observed upon the addition of G_{i1} and G_{i1}. This suggests that for the β2AR agonist, binding alone cannot fully stabilize the G protein bound conformation, as has been observed in previous fluorescence and double electron electron resonance spectroscopy experiments (8, 13). The structural changes stabilized by G_{i1} and G_{i1} are qualitatively the same for TM5, TM6, and TM7; however, our NMR studies and supporting MD simulations suggest that G_{i1} does not promote the formation of an alpha helix in ICL2.

Discussion

We applied NMR spectroscopy to monitor structural changes in the cytoplasmic surface of the unliganded β2AR and in response to an inverse agonist, an agonist, G_{i1} and G_{i1}. We have been unable to obtain crystal or cryo-EM structures of the apo-β2AR and agonist-bound β2AR due to its instability and possibly due to conformational heterogeneity. Our NMR studies provide further evidence for conformational heterogeneity as we observe more than one peak for K14^{7.39}K273^{6.35} and K328^{7.55} in apo and agonist-bound β2AR (Figs. 2–4). Agonist binding leads to changes in K14^{7.39} in ICL2, K26^{7.29}, and K27^{8.35} in TM6, and in K32^{7.55} in TM7; however, additional changes are observed upon the addition of G_{i1} and G_{i1}. This suggests that for the β2AR agonist, binding alone cannot fully stabilize the G protein bound conformation, as has been observed in previous fluorescence and double electron electron resonance spectroscopy experiments (8, 13). The structural changes stabilized by G_{i1} and G_{i1} are qualitatively the same for TM5, TM6, and TM7; however, our NMR studies and supporting MD simulations suggest that G_{i1} does not promote the formation of an alpha helix in ICL2.

Molecular Dynamics Simulations Showed Different ICL2 Behavior in the β2AR-G_{i1-EMPTY} and the β2AR-G_{i1-EMPTY} Complex. To investigate the dynamics at the receptor G protein interface, classical unbiased MD simulations of the β2AR-G_{i1-EMPTY} and the β2AR-G_{i1-EMPTY} complex were carried out. The β2AR-G_{i1-EMPTY} model was built based on the β2AR-G_{i1-EMPTY} complex (Protein Data Bank [PDB] ID code: 3SN6) (see Methods for more details) and as a result, ICL2 in the starting structures of both β2AR-G_{i1-EMPTY} and β2AR-G_{i1-EMPTY} complex are in an alpha-helical conformation. Three independent, 3-μs-long molecular dynamics (MD) simulations were carried. While the simulations are too short to observe unfolding of the secondary structure, the significantly higher rmsd of the ICL2 region of the β2AR-G_{i1-EMPTY} complex compared to the β2AR-G_{i1-EMPTY} complex suggests the starting alpha-helical structure of ICL2 is less stable in the β2AR-G_{i1-EMPTY} structure. Of note, such changes are not observed for ICL1, H8, nor G_{a}(a5) when both systems are compared (Fig. 6A–D). The only other structural elements at the receptor G protein interface with increased rmsd values are TM5 and TM6 (Fig. 6E and F), consistent with the high dynamics of their cytoplasmic ends observed by chemical shift analysis in β2AR-G_{i1-EMPTY} and in β2AR-G_{i1-EMPTY} complexes. A close inspection of the MD simulations results suggests a higher flexibility of K140^{34.52} and K147^{47.39} from ICL2 for the β2AR-G_{i1-EMPTY} compared to ICL2 from β2AR-G_{i1-EMPTY} as measured by the average rms fluctuation (RMSF) (SI Appendix, Fig. S10). The average RMSF value for K140^{34.52} is two times higher and for K147^{47.39} is six times higher in β2AR-G_{i1-EMPTY} than β2AR-G_{i1-EMPTY}. The MD simulations results are in agreement with the NMR experimental observation that ICL2 plays different roles when β2AR couples to G_{i1} or G_{i1}.

Methods

The receptors were expressed in S9 insect cells, purified by affinity column and size exclusion chromatography, and labeled by reductive methylation. NMR data were collected at 25 °C on a Bucker Avance 800-MHz spectrometer equipped with a cryoprobe. The 1H-13C HSQC spectra were recorded with spectral width of 11,160.71428 Hz in the 1H-dimension (w1) and 14,492.7536 Hz in the 13C-dimension (w2) centered at 46 ppm. For all spectra, 1024 × 256 complex points were recorded and a relaxation delay of 2 s was used to allow spin to relax back to equilibrium. MD simulations were performed using Gromacs simulation package. Further details are proved in SI Appendix.

Data Availability. All study data are included in the article and SI Appendix.

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