Allosteric coupling from G protein to the agonist–binding pocket in GPCRs

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G-protein-coupled receptors (GPCRs) remain the primary conduit by which cells detect environmental stimuli and communicate with each other1. Upon activation by extracellular agonists, these seven-transmembrane-domain-containing receptors interact with heterotrimeric G proteins to regulate downstream second messenger and/or protein kinase cascades1. Crystalllographic evidence from a prototypic GPCR, the β2-adrenergic receptor (β2AR), in complex with its cognate G protein, Gs, has provided a model for how agonist binding promotes conformational changes that propagate through the GPCR and into the nucleotide-binding pocket of the G protein α-subunit to catalyse GDP release, the key step required for GTP binding and activation of G proteins2. The structure also offers hints about how G-protein binding may, in turn, allosterically influence ligand binding. Here we provide functional evidence that G-protein coupling to the β2AR stabilizes a ‘closed’ receptor conformation characterized by restricted access to and egress from the hormone-binding site. Surprisingly, the effects of G protein on the hormone-binding site can be observed in the absence of a bound agonist, where G-protein coupling driven by basal receptor activity impedes the association of agonists, partial agonists, antagonists and inverse agonists. The ability of bound ligands to dissociate from the receptor is also hindered, providing a structural explanation for the G-protein-mediated enhancement of agonist affinity, which has been observed for many GPCR–G-protein pairs. Our data also indicate that, in contrast to agonist binding alone, coupling of a G protein in the absence of an agonist stabilizes large structural changes in a GPCR. The effects of nucleotide-free G protein on ligand-binding kinetics are shared by other members of the superfamily of GPCRs, suggesting that a common mechanism may underlie G-protein-mediated enhancement of agonist affinity.

Sequencing of the human genome revealed the magnitude of the GPCR superfamily, identifying over 800 genes encoding GPCRs, making this class of receptors the third-largest gene family2. Despite the varying nature of the chemical stimuli, which range from photons to small-molecule odorants and hormones to larger peptides and proteins, the generation of G-protein-mediated signals proceeds by a common mechanism. After activation, the receptor engages a heterotrimeric G protein and catalyses release of GDP from the G protein α-subunit (Gα). Intracellular GTP then binds the nucleotide-free G protein, allowing it to regulate downstream effectors (adenyl cyclase, phospholipase C, ion channels, and so on) to elicit cellular responses3. We recently used X-ray crystallography2, hydrogen–deuterium exchange mass spectrometry4 and electron microscopy5 to characterize an agonist–GPCR–G-protein ternary complex in the absence of nucleotide. These studies revealed dramatic conformational changes in the G protein that are stabilized by binding to agonist-activated receptor and provided insight into the mechanism by which GPCRs bind G proteins to promote nucleotide exchange. Here, we suggest an explanation for the allosteric communication that links the nucleotide-binding site on the G protein to the hormone-binding site on the receptor, with a focus on conformational changes in the extracellular face of the receptor that alter access to the hormone-binding site.

GPCR–G-protein interactions have historically been monitored using radioligand binding assays. Observations as early as the 1970s led to a progressive decrease in [3H]DHAP binding over time, which could be restored with excess GDP. Addition of apyrase to GDP-bound β2AR complexes led to a progressive decrease in [3H]DHAP binding over time, which could be restored with excess GDP. Addition of increasing concentrations of GDP enhances the rate and extent of [3H]DHAP binding to apyrase-treated β2AR complexes. Data are shown as mean ± standard error of the mean (s.e.m.) from n = 3 independent experiments performed in duplicate. b, Data are representative of three independent experiments.

Figure 1 | Guanine nucleotides influence antagonist binding to β2AR-Gs complexes. a, Binding of 2 nM [3H]DHAP to β2AR-Gs in the absence or presence of GDP. Addition of apyrase to GDP-bound β2AR-Gs led to a progressive decrease in [3H]DHAP binding over time, which could be restored with excess GDP. b, Addition of increasing concentrations of GDP enhances the rate and extent of [3H]DHAP binding to apyrase-treated β2AR-Gs complexes. a, Data are shown as mean ± standard error of the mean (s.e.m.) from n = 3 independent experiments performed in duplicate. b, Data are representative of three independent experiments.
suggested that G-protein coupling enhances agonist affinity for the receptor, and can be abolished by uncoupling the G protein from the receptor with guanine nucleotides. These and other data formed the basis for the ternary complex model of agonist–receptor–G-protein interactions. In this paradigm, the active state of the receptor is stabilized by both the agonist and G protein, and enhancement of agonist affinity arises owing to the positive cooperativity between agonist and G protein. However, using purified β2AR–Gs complexes, we observed peculiar binding characteristics of the antagonist [3H]dihydroalprenolol ([3H]DHAP) to β2AR (Fig. 1a). As illustrated, addition of GDP increases the observed binding of a saturating concentration of [3H] DHAP, whereas removal of GDP using a nucleotide lyase, apyrase, decreases [3H]DHAP binding. The apyrase-mediated decrease in [3H] DHAP binding is reversed upon addition of excess GDP, suggesting that the decrease is indeed due to the formation of nucleotide-free β2AR•Gs complexes. Removal of GDP from the β2AR•Gs complex relies on the constitutive activity of β2AR and the rapid hydrolysis (by apyrase) of GDP released from the α-subunit of Gs, Gsα. The nucleotide-free status of Gsα in these β2AR•Gs complexes was confirmed by rapid [35S]GTP–S binding kinetics (Extended Data Fig. 1). The observed deficit in [3H]DHAP binding to nucleotide-free β2AR•Gs is the result of slower [3H]DHAP association with β2AR (Fig. 1b and Extended Data Fig. 2). GDP enhances [3H]DHAP association in a concentration-dependent manner, with similar effects achieved by complete β2AR•Gs uncoupling with GTP–S. Although nucleotides do not significantly affect the affinity (dissociation constant (KD) of [3H]DHAP, their modulatory capacity is γ-phosphate-dependent since GTP–S is approximately tenfold more potent than GDP (Extended Data Fig. 3). Thus, β2AR bound to nucleotide-free G protein adopts a conformation characterized by restricted access to the hormone-binding site.

Crystallographic and pharmacological evidence suggests that the active conformation of the β2AR is stabilized by nucleotide-free Gs or by a single-chain camelid antibody raised against agonist-bound β2AR (nanobody Nb80) (Fig. 2a,b;11,12. As illustrated in Fig. 2b (and Extended Data Fig. 4a), Nb80 stabilizes a conformation of the β2AR that restricts [3H]DHAP association, similar to nucleotide-free Gs. Importantly, Nb80 also slows the association of full agonist [3H]formoterol (e), partial agonist [3H]CGP12177 (d), and inverse agonist [3H]carvedilol (e) to the β2AR.

f. Nb80 stabilizes the closed, active conformation and slows [3H]DHAP dissociation from the β2AR in a concentration-dependent manner.

b, f. Data are representative of three independent experiments. All other data are specific binding, shown as mean ± s.e.m. from n = 3 independent experiments performed in duplicate.
Figure 3 | Activation of the β₂AR closes the hormone-binding site. a, Stabilization of the β₂AR active conformation by Gs (or Nb80) brings the side chains of Phe193ECL2 and Tyr308β3.5 close to one another compared to their positions in structures in the absence of G protein. b, Closer view of the orthosteric site, highlighting Phe193ECL2 and Tyr308β3.5. Distances (in Å) between the hydroxyl on Tyr308β3.5 and 2-carbon on the phenyl ring of Phe193ECL2 are indicated. c, d, A surface view comparing the extracellular face of the β₂AR in inactive (c) or active (d) conformations, showing how G-protein-stabilized structural rearrangements occlude the hormone-binding site in the active state. e, f, Cutaway view illustrating closure of the hormone-binding site around the bound agonist in the active state. The inverse agonist carazolol is shown in orange, the agonist BI-167107 is shown in yellow.

and Supplementary Video 1). The binding of Gs or Nb80 to the β₂AR stabilizes a rearrangement of the cytoplasmic end of transmembrane domain 7 (TM7; Fig. 4a, b) that is accompanied by changes immediately above the ligand-binding site, as well as a change in the structure of the extracellular loop (ECL) between TM4 and TM5 (ECL2). In comparison to the inactive β₂AR, the structure of the β₂AR–Gs or β₂AR–Nb80 (or related Nb6B9)14 complex identifies two aromatic residues, Phe193β3.25 or ECL2 and Tyr308β3.35, that move approximately 2–2.5 Å closer to each other to form a lid-like structure over the orthosteric ligand-binding site. Lys305β3.32 also contributes to capping the orthosteric site by trading its salt bridge15 with Asp192ECL2 for an interaction with the backbone carbonyl of Phe193ECL2 (Fig. 4c). These structural changes are stabilized in the active forms of the β₂AR bound to either the ultra–high-affinity agonist BI-167107 or the smaller, low-affinity agonist adrenaline15, and formation of this ‘lid’ would be expected to sterically obstruct both ligand association and dissociation.

To validate this structural model, we tested whether a residue smaller than tyrosine could modify the capacity of Nb80 to slow ligand association. Mutation of Tyr308β3.35 to alanine, previously shown to lower agonist affinity for the β₂AR16, significantly diminishes the capacity of Nb80 to slow the association of [³H]DHAP and even the agonist [³H]formoterol (Extended Data Fig. 5a), as suggested by recent molecular dynamics simulations17. Interestingly, and in contrast to [³H]DHAP association, pre-incubation with 10μM Nb80 also enhances the extent of [³H]formoterol binding in the Y308A mutant. Eliminating barriers that impair access to the orthosteric site (for example, Y308A) allows the agonist to at least enter the receptor, where it can stabilize nanobody binding. The enhancement, therefore, is a reflection of the capacity of the agonist [³H]formoterol to cooperatively stabilize Nb80 binding and vice versa, and concomitantly slow the dissociation of the bound agonist (Extended Data Fig. 5d). The data also suggest that while Tyr308β3.35 markedly limits access to the orthosteric site, other residues may work in concert with Tyr308β3.35 to form a lid over the hormone-binding site.

Figure 4 | Allosteric communication between the β₂AR G-protein- and hormone-binding sites. a, In the β₂AR active state (cyan), the cytoplasmic end of TM6 moves away from the receptor core by ~14 Å relative to its position in the inactive-state structure, allowing for an inward movement of TM7. b, Rotation of TM7 allows Tyr326β7.53 (of the highly conserved NPxxY motif) to fill the space vacated by the conserved aliphatic residue Ile278β6.40. c, The rotation of TM7 repositions Tyr308β3.35 and Lys305β3.32. This conformational change allows Lys305β3.32 to coordinate the backbone carbonyl of Phe193ECL2, stabilizing its movement towards Tyr308β3.35 to form a lid over the hormone-binding site.

It is noteworthy that the movement of Phe193ECL2 and Tyr308β3.35 is not fully observed in the crystal structure of the β₂AR bound to an agonist alone18, nor in the inactive-state structure of the β₂AR bound to the agonist isoprenaline19 (Extended Data Fig. 6 and Supplementary Videos 1, 2). Binding of G protein or G-protein mimetic (nanobody) is sufficient to stabilize the closed, active conformation since their effects on ligand-binding kinetics (as in Figs 1 and 2) are agonist-independent. An agonist may enhance G-protein engagement but poorly stabilizes the closed, active conformation by itself. Additionally, the data presented here suggest that formation of the closed, active conformation stabilized by the nucleotide-free G protein can occur owing to basal receptor activity, in keeping with predictions of more recent models of GPCR pharmacology such as the extended and cubic ternary complex models20,21 (see Supplementary Discussion). Moreover, conformational changes stabilized by the nucleotide-free G protein influence not only agonist binding, but ligand binding in general, implying that the role of nucleotides needs to be included in an updated version of ternary complex model.
positive modulator LY2119620 utilizes residues that form the lid in the active, closed conformation as described here, as the floor of the vestibule. Stabilization of this closed conformation may therefore be an important aspect on the differentiation between positive allosteric modulators, which enhance agonist binding and activation, and negative allosteric modulators, which decrease agonist binding.

We provide pharmacological and biochemical evidence suggesting that the closed, active conformation of GPCRs is stabilized by the nucleotide-free G protein, allowing G proteins to influence passage of ligands to the orthosteric-binding site. The dramatic effect of G proteins on either ligand association or dissociation is consistent with, and in fact validates, structural models generated from X-ray crystallography in which G-protein coupling on the intracellular face of the receptor allosterically influences the structure of the extracellular face. Agonist or hormone binding enhances G-protein engagement, whereby formation of the active receptor conformation is accompanied by nucleotide loss from the G protein. Therefore, the capacity of G proteins to enhance agonist-binding affinity is structurally and energetically linked to the agonist's capacity to promote nucleotide loss from Go.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Figure 5 | Basis for G-protein-dependent high-affinity agonist binding.** Agonist binding promotes the G-protein–receptor (R) interaction and GDP release from the G-protein heterotrimer (GαS (α) Gβγ (βγ)). In this nucleotide-free state, the C-terminal helix of Gα remains embedded in the receptor core, stabilizing the conformational changes at both the intracellular and extracellular faces of the receptor. At the extracellular side, the orthosteric ligand-binding site closes around the bound agonist, sterically opposing agonist dissociation and thereby enhancing the observed affinity. Constitutive (basal) receptor activity may also activate the G protein, releasing GDP and thereby stabilizing the closed conformation of the receptor in the absence of an agonist. See also Extended Data Fig. 10.

The capacity of G proteins to stabilize a closed receptor conformation explains the poorly defined GTP–S–mediated increase in radio-labelled antagonist binding observed with several GPCRs, including muscarinic, α-adrenergic, adenosine and opioid receptors (as in Extended Data Figs 7 and 8). We analysed the behaviour of the M2 muscarinic acetylcholine receptor (M2R) and the μ-opioid receptor (MOPr) to determine whether GTP–S–mediated uncoupling relieves a G-protein–stabilized closed conformation. We focused on these receptors since structural models are available for both inactive and active conformations, and to determine whether the mechanism we propose for the β2AR is shared among other GPCRs. The active-state structure of the M2R, in particular, revealed similar conformational changes to the β2AR in that a lid-like structure is formed above the orthosteric site (see Supplementary Videos 4 and 5). Although the structural changes are not identical, the effect of G proteins (or nabenoids) on the association and dissociation of ligands at the orthosteric sites is shared among the β2AR, M2R and MOPr (Extended Data Fig. 9 and Supplementary Video 3), suggesting that the allosteric effects of G proteins on orthosteric agonists may be manifested by conceptually common mechanisms. More discussion of the details and implications can be found in Supplementary Discussion. Additionally, many recent studies have focused on the allosteric effect of sodium ions on class A GPCR ligand binding and signalling. Outward movement of TM6 during receptor activation collapses the sodium-binding pocket in many class A GPCRs, thus it appears that loss of bound sodium is necessary for G proteins to stabilize a closed, active receptor conformation.

The formation of the closed conformation is also of particular interest for the development of allosteric modulators targeting class A GPCRs. Most allosteric-modulator-binding sites have focused on the extracellular vestibule located above the orthosteric ligand-binding sites. For the muscarinic M2R for example, the potent M2R allosteric
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Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.K.S. (rsunahara@ucsd.edu).
METHODS

Large-scale purification of the β2AR. β2AR bearing an N-terminal Flag tag and C-terminal 10×His was expressed in SF9 cells (Invitrogen) and purified as previously described2. Expression and purification of G protein and nanobodies. Gs and Go heterotrimer were expressed in HighFive (Invitrogen) insect cells coinfected with baculavirus and purified by chromatography on Ni-NTA, MonoQ, and Superdex 200 resin, as previously described3. Nanobodies were expressed in Escherichia coli and purified as previously described4,5. Membrane preparations. HEK293T cells (ATCC) were used for small-scale expression and purification of β2AR and mutants. Cells were grown in DMEM plus 10% FBS to ~70% confluency, then transfected with monomeric yellow fluorescent protein (mYFP)-β2AR (pCMV5, 6.1 μg DNA per 10-cm plate) using Lipofectamine 2000. Cells were harvested 48–48 h post-transfection in ice-cold lysis buffer buffer (50 mM HEPES, pH 8.0, 65 mM NaCl, 1 mM EDTA, 35 μg/ml−1 phenylmethylsulfonyl fluoride, 32 μg/ml−1 each tosyl-l-phenylalanine-chloromethylketone and tosyl-l-lysine-chloromethylketone, 3.2 μg/ml−1 leupeptin, 3.2 μg/ml−1 ovomucoid trypsin inhibitor). The cell suspension was sonicated using a Branson Sonifier and centrifuged for 20 min at 25,000g. The pellet was resuspended in wash buffer (50 mM HEPES, pH 8.0, 100 mM NaCl with protease inhibitors listed earlier) using a Dounce homogenizer, then centrifuged for 20 min at 25,000g. The pellet was resuspended and homogenized in minimal wash buffer and the volume was adjusted to reach a final protein concentration of 5 mg ml−1 as measured by the Bradford protein assay. Membranes were frozen by slowly pouring into liquid nitrogen and stored at −80°C until use.

Enrichment of β2AR and β2AR(Y308A) from HEK293T cells. Frozen membranes were thawed on ice and NaCl, MgCl2, and GTP•S were added to reach final concentrations of 300 mM, 1 mM and 10 μM, respectively. Timolol was then added to a final concentration of 1 μM and the membranes were incubated for 10 min on ice. Receptors were solubilized for 1 h at 4°C in the presence of 1% dodecylmaltoside (DDM) and 0.1% cholesterol hemisuccinate (CHS). After centrifugation for 20 min at 25,000 × g and the membranes were thawed on ice and NaCl, MgCl2, and GTP•S to remove bound timolol. Receptor was eluted in 50 mM HEPES, pH 8.0, 65 mM NaCl with protease inhibitors listed earlier) using a Dounce homogenizer, then centrifuged for 20 min at 25,000g. The pellet was resuspended in wash buffer (50 mM HEPES, pH 8.0, 100 mM NaCl with protease inhibitors listed earlier) using a Dounce homogenizer, then centrifuged for 20 min at 25,000g. The pellet was resuspended and homogenized in minimal wash buffer and the volume was adjusted to reach a final protein concentration of 5 mg ml−1 as measured by the Bradford protein assay. Membranes were frozen by slowly pouring into liquid nitrogen and stored at −80°C until use.

Receptor reconstitution into rHDL particles. Reconstitutions were performed as described3, with the amount of receptor added never exceeding 20% of the total reaction volume. For samples that contained Gs, the purified heterotrimer was added to the preformed β2AR–rHDL particles, incubated for 2 h at 4°C, and BioBeads (Bio-Rad) were used to remove the added detergent. Nucleotide-free Gs;β2AR complex was prepared by incubating β2AR–Gs–rHDL particles with apyrase in the presence of 1 mM MgCl2 for 30 min at room temperature, or alternatively, 2 h at 4°C. If needed, the sample was passed through a Superdex 200 gel filtration column to remove free nucleotide and apyrase.

Radioligand association experiments using rHDL particles. All assays were performed in Tris-buffered saline (TBS; 25 mM Tris•HCl, pH 7.4, 135 mM NaCl, 2.7 mM KCl) with a final concentration of 0.05% w/v bovine serum albumin (BSA). Reaction components were mixed and pre-incubated at room temperature (see later) before the addition of radioligand to initiate the association time course. Aliquots were withdrawn at the indicated times and filtered over Whatman GF/B filters pre-soaked in 0.3% w/v polyethyleneimine. Filters were washed with ice-cold TBS, dried, and subjected to liquid scintillation counting on a TopCount NXT (Perkin-Elmer). Bound ligand never exceeded 10% of the total ligand added. Kinetic binding experiments with [3H]DHAP and Nb80, β2AR•rHDL. For association experiments, receptor in rHDL was pre-incubated with varying concentrations of Nb80 and the reaction was started by addition of 5 nM [3H]DHAP (Perkin Elmer). For dissociation experiments, the samples were first incubated with 5 nM [3H]DHAP for 30 min, followed by incubation with varying Nb80 concentrations for 30 min. The reaction was started by adding 50 μM cold apronanol. Non-specific binding was determined in the presence of 10 μM (+/-) propranolol.

Binding experiments with [3H]DHAP and Gs;β2AR nucleotide-free complexes. For association experiments, gel-filtered samples of apyrase-treated Gs;β2AR–rHDL particles were incubated with 5 nM [3H]DHAP to bind any receptor that was not complexed with Gs. The experiment was started by adding varying amounts of either GDP or GTP•S. For 'equilibrium' binding experiments, samples were incubated with all the indicated components at room temperature for 90 min before filtration. Non-specific binding was determined in the presence of 10 μM (+/-) propranolol. [3H]formoterol association to β2AR. β2AR–rHDL was incubated with the indicated concentrations of Nb80 for 30 min at room temperature. [3H]formoterol (Perkin Elmer) was added to reach 10 nM final concentration. These assays also contained 1 mM ascorbic acid in the reaction buffer. Non-specific binding was determined in the presence of 10 μM (+/-) propranolol. [3H](-)–CGP-12177 association to β2AR. β2AR–rHDL was incubated with the indicated concentrations of Nb80 for 30 min at room temperature. [3H](-)–CGP-12177 (Perkin Elmer) was added to reach 1 nM final concentration. Non-specific binding was determined in the presence of 10 μM (+/-) propranolol. [3H]carvedilol association to β2AR. Owing to high amounts of non-specific [3H]carvedilol (American Radiolabelled Chemicals) binding both to BSA and to the glass fibre filters typically used for separation, β2AR–rHDL was diluted into empty rHDL particles rather than into a 5× BSA solution (0.25% w/v BSA in TBS buffer) before addition to the assay mix. Using empty rHDL in place of BSA was critical for maintaining sample recovery from the assay plate while improving the signal-to-noise ratio of the assay. The receptor was incubated with the indicated concentrations of Nb80 for 15 min at room temperature, then for 30 min at 4°C. [3H]carvedilol was added to reach a 1 nM final concentration. Aliquots were withdrawn at the indicated time points and bound ligand was isolated using gel filtration on Sephadex G75 resin. Non-specific binding was determined in the presence of 10 μM (+/-) propranolol.

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Extended Data Figure 1 | Confirmation of nucleotide removal from β₂AR-Gs by apyrase. Gs and Flag-tagged β₂AR were reconstituted in rHDL and treated with the non-specific nucleotide lyase, apyrase. Samples were applied to an anti-Flag affinity resin to remove products of the GDP degradation (GMP and Pi). Samples were incubated with 100 nM [³⁵S]GTPγS at room temperature. At various times, samples were subjected to rapid filtration through glass fibre filters (GF/B) followed by 10 volumes of ice-cold buffer washes containing 10 μM GDP. Filters were dried and subjected to liquid scintillation counting (Top-Count, Perkin-Elmer).

To a first approximation, the rapid binding event suggests that the complex is empty of nucleotide, based on the limited temporal resolution of this mixing and filtration technique. [³H]DHAP and [³⁵S]GTPγS binding to the reconstituted complex yields a final R:G ratio of 1:0.95, suggesting that up to 95% of the β₂AR-rHDL particles contain a single functional G protein. This suggests that only those G proteins associated with the β₂AR will bind [³⁵S]GTPγS within this time frame in the absence of receptor agonists. Data are shown as mean ± s.e.m. from n = 3 independent experiments performed in duplicate.
Extended Data Figure 2 | GDP accelerates [$^3$H]DHAP binding to $\beta_2$AR•Gs. a, Time course monitoring [$^3$H]DHAP association to apyrase-treated $\beta_2$AR•Gs complexes in the presence of varying GDP concentrations. GDP increases both the observed association rate constant and the maximum binding of [$^3$H]DHAP. b, Concentration–response curve showing enhancement of the observed [$^3$H]DHAP association rate constant by GDP (half-maximum effective concentration ($EC_{50}$) = 181 ± 66 nM). All data are shown as mean ± s.e.m. from $n = 3$ independent experiments performed in duplicate.
Extended Data Figure 3 | Effect of guanine nucleotides on $[^3\text{H}]\text{DHAP}$ binding to $\beta_2\text{AR}•\text{Gs}$. 

**a**, In saturation binding assays, addition of GTP$\gamma$S to apyrase-treated $\beta_2\text{AR}•\text{Gs}$ complexes increased the observed maximal binding, $B_{\text{max}}$, for $[^3\text{H}]\text{DHAP}$ without significantly altering $K_d$ (control: $B_{\text{max}} = 5.5 \pm 0.52$ fmol, $K_d = 0.88$ nM; +GTP$\gamma$S: $B_{\text{max}} = 16.6 \pm 1.9$ fmol, $K_d = 0.56$ nM). **b**, Both GDP and GTP$\gamma$S could enhance maximal $[^3\text{H}]\text{DHAP}$ binding in a concentration-dependent manner (GDP log(EC$_{50}$) = $-6.42 \pm 0.12$, or EC$_{50}$ ≈ 386 nM; GTP$\gamma$S log(EC$_{50}$) = $-7.45 \pm 0.16$, or EC$_{50}$ ≈ 35 nM). All data are shown as mean ± s.e.m. from $n = 3$ independent experiments performed in duplicate.

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Extended Data Figure 4 | Effect of Nb80 on antagonist binding to the β2AR. a, Association of [3H]DHAP is progressively slowed after pre-incubation of the β2AR with increasing concentrations of Nb80. b, If [3H]DHAP is allowed to first equilibrate with the β2AR, Nb80 slows [3H]DHAP dissociation from β2AR in a concentration-dependent manner. c, Owing to the dramatic slowing of [3H]DHAP binding kinetics, Nb80 (but not a control nanobody, Nb30, which has no effect on agonist affinity for β2AR) seems competitive with [3H]DHAP if insufficient time is given to reach equilibrium. Data shown are from assays incubated for 90 min at room temperature. All data are shown as mean ± s.e.m. from n = 3 independent experiments performed in duplicate.
Extended Data Figure 5 | Y308A mutation abolishes the rate-slowing effects of Nb80. 

(a, b) Time course of [3H]DHAP binding to wild-type (WT) β2AR (a) or β2AR(Y308A) (b) after pre-incubation of receptor with Nb80. Nb80 significantly slowed [3H]DHAP association to wild-type β2AR (−Nb80 observed rate constant, \(k_{\text{obs}} = 0.45 \pm 0.05 \text{ min}^{-1}\) or association half-time, \(t_{50} = 1.5 \pm 0.2 \text{ min}\), +Nb80 \(k_{\text{obs}} = 0.20 \pm 0.03 \text{ min}^{-1}\) or \(t_{50} = 3.5 \pm 0.5 \text{ min}; P = 0.011\) by an unpaired two-tailed \(t\)-test), but less effectively slowed [3H]DHAP association to β2AR(Y308A) (−Nb80 \(k_{\text{obs}} = 0.50 \pm 0.06 \text{ min}^{-1}\) or \(t_{50} = 1.4 \pm 0.2 \text{ min}\), +Nb80 \(k_{\text{obs}} = 0.32 \pm 0.01 \text{ min}^{-1}\) or \(t_{50} = 2.2 \pm 0.1 \text{ min}\); \(P = 0.05\) by an unpaired two-tailed \(t\)-test). All data are shown as mean ± s.e.m. from \(n = 4\) (−Nb80) or \(n = 3\) (+Nb80) independent experiments performed in duplicate. 

(c, d) Time course of [3H]formoterol binding to wild-type β2AR (c) or β2AR(Y308A) (d) after pre-incubation of receptor with Nb80. Nb80 slowed [3H]formoterol association to wild-type β2AR (0.1 μM Nb80 \(k_{\text{obs}} = 0.68 \pm 0.13 \text{ min}^{-1}\) or \(t_{50} = 1.0 \pm 0.2 \text{ min}\), 10 μM Nb80 \(k_{\text{obs}} = 0.27 \pm 0.05 \text{ min}^{-1}\) or \(t_{50} = 2.6 \pm 0.5 \text{ min}; P = 0.031\) by an unpaired two-tailed \(t\)-test). However, with β2AR(Y308A), Nb80 had little effect on the observed association rate constant but enhanced the amount of [3H]formoterol bound (0.1 μM Nb80 \(k_{\text{obs}} = 0.37 \pm 0.11 \text{ min}^{-1}\) or \(t_{50} = 1.9 \pm 0.6 \text{ min}\) with a plateau of 10.1 ± 0.8 fmol, 10 μM Nb80 \(k_{\text{obs}} = 0.53 \pm 0.13 \text{ min}^{-1}\) or \(t_{50} = 1.3 \pm 0.4 \text{ min}\) with a plateau of 21.3 ± 1.2 fmol; unpaired two-tailed \(t\)-test of the \(k_{\text{obs}}\) values showed \(P = 0.4\)). All data are shown as mean ± s.e.m. from \(n = 4\) independent experiments performed in duplicate.
Extended Data Figure 6 | The closed conformation stabilized by agonist and G protein (or mimic). Illustrated are the crystal structures of agonist-versus inverse-agonist-bound $\beta_2$AR (cyan) and $\beta_1$AR (yellow), where only $\beta_2$AR is bound to G protein. Similarly, the MOPr (orange) adopts a closed conformation upon binding the G-protein surrogate, Nb39. $\beta_2$AR, PDB accession 2RH1; $\beta_2$AR•Gs, PDB accession 3SN6; $\beta_1$AR, PDB accession 2YCW; $\beta_1$AR•iso, PDB accession 2Y03; MOPr, PDB accession 4DKL; MOPr–Nb39, PDB accession 5C1M; M2R, PDB accession 3UON; M2R–Nb9–8, PDB accession 4MQS.
Extended Data Figure 7 | Effect of guanine nucleotides on [3H] antagonist binding are also seen in competition binding assays. a, Agonist (isoproterenol) competition binding using apyrase-treated β2AR•Gs complexes shows the characteristic G-protein-dependent shift in agonist affinity, along with a dramatic increase in total [3H]DHAP binding, upon the addition of 10 μM GTPγS. b, Normalization of the data from a yields a plot representative of what is commonly reported in the literature. c, Similar to the β2AR, agonist (morphine) competition binding using MOPr•Go complexes shows the characteristic G-protein-dependent shift in agonist affinity, along with a dramatic increase in total [3H]DPN binding, upon the addition of 10 μM GTPγS. d, Normalization of the data from c. All data are shown as mean ± s.e.m. from n = 3 independent experiments performed in duplicate.
Extended Data Figure 8 | The MOPr and M2R behave similarly to the β2AR when bound to nucleotide-free G protein or an active-state-stabilizing nanobody. a, After apyrase treatment of M2R•Go complexes, addition of 10μM GTPγS enhances association of [3H]N-methylscopolamine ([3H]NMS) to M2R (vehicle $k_{obs} = 0.32 \pm 0.02$ min$^{-1}$ or $t_{1/2} = 2.2 \pm 0.1$ min; +GTPγS $k_{obs} = 0.54 \pm 0.02$ min$^{-1}$ or $t_{1/2} = 1.3 \pm 0.1$ min; $P = 0.002$ by an unpaired two-tailed t-test). Data are shown as mean ± s.e.m. from $n = 3$ independent experiments performed in duplicate. Addition of GDP was also able to increase the rate of [3H]NMS binding (inset; log (EC50) = 6.91 ± 0.18 or EC50 ≈ 123 nM; mean ± s.e.m. from $n = 2$ independent experiments performed in duplicate).
b, Pre-treatment of M2R with either 10μM (black circles) or 100μM (red squares) Nb9-8 (ref. 27) impairs association of [3H]iperoxo to M2R (10μM Nb9-8 $k_{obs} = 0.68 \pm 0.09$ min$^{-1}$ or $t_{1/2} = 1.0 \pm 0.2$ min; 100μM Nb9-8 $k_{obs} = 0.25 \pm 0.04$ min$^{-1}$ or $t_{1/2} = 2.8 \pm 0.5$ min; $P = 0.04$ by an unpaired two-tailed t-test). Data are shown as mean ± s.e.m. from $n = 3$ (10μM Nb9-8) or $n = 2$ (100μM Nb9-8) independent experiments performed in duplicate.
c, Addition of 10μM GTPγS to apyrase-treated MOPr•Go complexes hastened association of the antagonist [3H]diprenorphine ([3H]DPN) to MOPr (apyrase $k_{obs} = 0.06 \pm 0.02$ min$^{-1}$ or $t_{1/2} = 9.8 \pm 1.3$ min; +GTPγS $k_{obs} = 0.12 \pm 0.01$ min$^{-1}$ or $t_{1/2} = 5.6 \pm 0.6$ min; $P = 0.1$ by an unpaired two-tailed t-test). The effect of nucleotide-free G protein was recapitulated by pre-incubating MOPr with Nb39 (ref. 28) (inset; control $k_{obs} = 0.13 \pm 0.01$ min$^{-1}$, +100μM Nb39 $k_{obs} = 0.07 \pm 0.02$ min$^{-1}$). Data are shown as mean ± s.e.m. from $n = 2$ (MOPr•Go) or $n = 3$ (MOPr + Nb39) independent experiments performed in duplicate.
Extended Data Figure 9 | The extracellular regions in the active conformations of peptide hormone/agonist receptors MOPr and NTS-R1. Illustrated are the crystal structures of the inactive and active (or partially active neurotensin receptor 1, NTS-R1) conformations of the MOPr and NTS-R1 from the top or extracellular view of the receptor. The surface rendering highlights residues or structure on the extracellular face that change upon receptor activation (circled). The MOPr in its inactive conformation (purple) is compared to the Nb39-bound (G-protein mimic) form in blue. Similarly, the inactive NTS-R1 (ref. 33) (green) is compared with a mutant NTS-R1 (ref. 34) that adopts a partially active conformation (orange). MOPr, PDB accession 4DKL; MOPr-Nb39, PDB accession 5C1M; NTS-R1, PDB accession 4GRV; active-like NTS-R1, PDB accession 4XEE.
Extended Data Figure 10 | Model of G-protein-dependent high-affinity agonist binding. **a**, **b**, As in Fig. 5, nucleotide-free G-protein-stabilized family A GPCRs experience alterations in the extracellular face of the receptor, thus affecting the orthosteric-binding site. In a monoamine receptor such as the β2AR, G-protein binding and GDP loss accompanies the stabilization of a closed, active conformation of the receptor, as in **a**. **b**, For family members such as MOPr or NTS-R1, where the peptide hormones/agonists are considerably larger, the influence of the G-protein-mediated changes in the extracellular domain structure result in similar effects on orthosteric ligand dissociation. Rather than closing over the orthosteric site as with monoamine receptors as in **a**, the extracellular face may contain structures and residues that ‘pinch’ the larger ligands.