Single-molecule analysis of ligand efficacy in β2AR–G-protein activation

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G-protein-coupled receptor (GPCR)-mediated signal transduction is central to human physiology and disease intervention, yet the molecular mechanisms responsible for ligand-dependent signalling responses remain poorly understood. In class A GPCRs, receptor activation and G-protein coupling entail outward movements of transmembrane helix 6 (TM6). Here, using single-molecule fluorescence resonance energy transfer imaging, we examine TM6 movements in the β2 adrenergic receptor (β2AR) upon exposure to orthosteric ligands with different efficacies, in the absence and presence of the Gα heterotrimer. We show that partial and full agonists differentially affect TM6 motions to regulate the rate at which GDP-bound β2AR–G complexes are formed and the efficiency of nucleotide exchange leading to Gα activation. These data also reveal transient nucleotide-bound β2AR–G species that are distinct from known structures, and provide single-molecule perspectives on the allosteric link between ligand- and nucleotide-binding pockets that shed new light on the G-protein activation mechanism.

GPCRs regulate cellular responses to neurotransmitters and hormones and act as ligand-regulated guanosine nucleotide exchange factors (GEFs) for heterotrimeric G proteins1. Ligand efficacy has historically referred to the capacity of a molecule to elicit a specific physiological response downstream of receptor activation1,2. Although an important parameter in drug development, the molecular basis of efficacy with respect to the effect of a ligand on GPCR structure, dynamics and G-protein coupling remains poorly understood.

β2AR, a paradigmatic, class A GPCR, couples preferentially to the heterotrimeric G protein, consisting of Gαs, Gβγ and Gβγ (Fig. 1a). Investigations of the β2AR activation mechanism have been enabled by synthetic ligands with efficacy profiles ranging from inverse agonists that suppress basal activity, and neutral antagonists that prevent agonist-induced activation, to partial and full agonists that differentially promote receptor-mediated Gα activation2. Recent crystallographic structures of distinct class A GPCRs in both inactive and active states6–9 revealed that the largest conformational change associated with their activation is an outward movement of the cytoplasmic end of TM6 (ref. 6). In the nucleotide-free β2AR–G complex, TM6 is stabilized in an outward configuration by insertion of the C-terminal α helix of Gαs into a pocket formed by the cytoplasmic ends of TM3, TM5 and TM6 and intracellular loop 2 (ICL2).

Ensemble techniques, including fluorescence10, electron paramagnetic resonance (EPR)11 and nuclear magnetic resonance (NMR) spectroscopy11,12 reveal that even the most potent agonists fail to fully stabilize β2AR in its activated conformation in the absence of G protein the cytoplasmic face (red mesh) of β2AR (PDB code 3SN6). GTP binding causes Gαs (PDB code 1AZT) and Gβγ subunit (not shown) to separate. b. Ligand efficacy profiles determined using a GTP turnover assay (Supplementary Methods; error bars denote s.e.m., three replicates). ADR, adrenaline; ALP, alprenolol; BI, BI-167107; CLEN, clenbuterol; CZ, carazolol; ISO, isoproterenol; SALB, salbuterol; SALM, salmeterol.

Figure 1 | High-resolution perspective of β2AR–Gα activation.

a. Labelling sites N148C (magenta) in TM4 and L266C (green) in TM6 (blue) are shown on the inactive β2AR structure (Protein Data Bank (PDB) accession 2BH1). Agonist activation leads to outward TM6 displacement (~14 Å) and Gα coupling (shown GDP-bound Gα; PDB code 1GP2). Within the GDP-free complex the α helix (red) of Gαs (wheat) engages

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or stabilizing nanobodies. The molecular basis of ligand efficacy may therefore be defined by changes in receptor dynamics and conformation that impact the probability of G-protein coupling, productive nucleotide exchange and subsequent dissociation. Hence, we used total internal reflection fluorescence (TIRF) single-molecule fluorescence resonance energy transfer (smFRET) imaging to track TM6 movements in β2AR bound to ligands with distinct efficacy profiles to determine the effects on receptor structure, dynamics, and G-protein coupling.

Site-specific labelling of β2AR

We site-specifically attached donor and acceptor fluorescent probes at the cytoplasmic ends of TM6 (L266C—6.25) and TM4 (N148C—4.0), respectively, within a full-length, minimal cysteine β2AR mutant (Fig. 1a, Supplementary Methods). This construct (β2Δ6-148C/266C) exhibits wild-type ligand binding and Gs coupling (Extended Data Fig. 1a–c).

Given the relatively small size of β2AR (~34 Å lateral dimension) and the anticipated displacement of TM6 (~14 Å) upon activation6 (Fig. 1a), β2Δ6-148C/266C was labelled with an optimized Cy3B and Cy7 fluorophore pair (Cy3B* and Cy7*; Extended Data Figs 2a, b and 3a, b, Supplementary Methods), which exhibit high quantum yields and a small R0 value (~50.7 Å) (Extended Data Fig. 2a–e) and should be relatively insensitive to chemical environment13. Labelled receptors showed wild-type activities with respect to both antagonist and agonist binding (Extended Data Fig. 1a, b).

Ligand-induced TM6 displacement

The effects of saturating concentrations of nine ligands with distinct efficacies (Fig. 1b, Extended Data Fig. 1d, e) were examined by imaging Cy3B*/Cy7*-labelled β2Δ6-148C/266C immobilized with an M1 Fab fragment (Fig. 2a, b, Extended Data Figs 3c and 4a, b). In the presence of the neutral antagonist alprenolol, and the inverse agonists carazolol and ICI-118,151 (ICI), β2AR exhibited indistinguishable population FRET efficiencies centred at 0.74 ± 0.01 (mean of biological replicates ± s.d.) and full-width at half-maximum height (FWHM) values of 0.1 ± 0.01 (Fig. 2b). Inspection of individual trajectories from alprenolol-, carazolol- and ICI-bound receptors revealed relatively stable fluorescence and FRET (Extended Data Fig. 4a). Similar results were obtained when β2AR was immobilized via biotinylated alprenolol (Extended Data Fig. 3d, e). These data predict an average inter-dye distance of approximately 42 Å (Extended Data Fig. 2d–f), in good agreement with molecular dynamics simulations (Extended Data Fig. 5a, b).

The unliganded (apo) β2AR exhibited mean FRET and FWHM values similar to that of alprenolol, carazolol and ICI (Fig. 2b). In the presence of the partial agonists clenbuterol, salmeterol or salbutamol, β2AR exhibited modestly lower, and more broadly distributed FRET values (0.70–0.72; FWHM = 0.15) (Fig. 2b). Reductions in FRET were more pronounced in the presence of the full agonists adrenaline, isoprotenerol and BI-167107, where the mean FRET value shifted to approximately 0.64 (FWHM = 0.15) (Fig. 2b). In agreement with radioligand-binding studies (Extended Data Fig. 1e), smFRET experiments showed that adrenaline exhibited a maximum effective concentration (EC50) of 0.48 ± 0.09 μM (mean of biological replicates ± s.e.m.) (Fig. 2c, Extended Data Fig. 3f). Hence, we infer that these adrenaline-induced FRET changes (ΔFRETapo/adrenaline = 0.1), corresponding to an increase in average inter-dye distance of approximately 4 Å (Extended Data Fig. 2e), reflect those of a fully functional receptor.

While the approximate 14 Å outward TM6 movement observed in fully activated β2AR (Fig. 1a; Extended Data Fig. 5) is anticipated to yield a FRET change of roughly 0.3 (Extended Data Fig. 2e), inspection of individual FRET trajectories revealed only rare fluctuations (around 1 min−1) of this amplitude (Extended Data Fig. 4a). However, correlation analyses14,15 revealed clear signatures of anticorrelated fluorescence within the ensemble of individual molecules (Fig. 2d–f, Extended Data Fig. 4c–e, Supplementary Methods), indicating fast (>10 s−1), reversible TM6 movements. By this measure, more rapid TM6 dynamics (more negative mean correlation) were observed in agonist-bound samples than antagonist-bound samples (Fig. 2e, f). Simulations revealed that the observed fluorescence correlation and FRET distributions could be recapitulated by TM6 deflections to lower FRET states at rates of 100–500 s−1 (Extended Data Fig. 4d, e). Previous reports of slower TM6 movements (~0.5–5.0 s−1)11,16 may reflect differences in experimental conditions or the nature of their probes, which detect changes in environment, not distance. Hence, we conclude that the distinct FRET values observed for each agonist reflect differences in the underlying rates and/or amplitudes of TM6 motions into, and out of, active-like conformations, which are time-averaged at the present imaging resolution.

The rate of β2AR–Gs complex formation

To ascertain directly the extent to which the ligand-induced changes observed in smFRET correlate with the coupling efficacy of β2AR to G protein, labelled β2AR (1 nM) was incubated with Gs (8 μM) and apyrase (0.2 nM). Individual β2AR molecules were subsequently imaged via M1 immobilization to determine the extent of β2AR–Gs complex formation (Fig. 3a).

In the presence of carazolol and ICI, β2AR exhibited FRET behaviours that were indistinguishable from those observed in the
absence of the Gs heterotrimer (Figs 2b, 3b). By contrast, agonist-activated β2AR complexes exhibited a distinct low-FRET (~0.4) state with similar FWHM values (~0.14) (Fig. 3b). This low-FRET value reflects an inter-dye distance of approximately 55 Å (Extended Data Fig. 2e), in close agreement with molecular dynamics simulations of the β2AR-Gs complex (Extended Data Fig. 5a–c). Analogous low-FRET states were observed when agonist-activated receptor was immobilized via a biotinylated Gs heterotrimer (Extended Data Fig. 6a–d). The proportion of receptors exhibiting low FRET values (Fig. 3b), as well as the fraction of time that individual receptors occupied low-FRET states (Extended Data Fig. 7a), correlated with ligand efficacy (Fig. 1b). Consistent with the known basal activity of β2AR17, low levels (5–20%) of Gs coupling were evidenced in the absence of ligand and in the presence of alpronolol (Fig. 3b, Extended Data Fig. 7a).

To examine the role of ligand efficacy on the rates of β2AR–Gs complex formation, M1-immobilized β2AR (Fig. 3c) was imaged in the presence of 30μM GDP at increasing Gs concentrations, where reversible transitions between high- and low-FRET states could be quantified using a two-state hidden Markov Model (Fig. 3d, Supplementary Methods). Consistent with a bimolecular reaction entailing at least one ligand-dependent, rate-determining process that precedes complete Gs coupling, we found that rates of low-FRET state formation increased with Gs concentration and then plateaued (Fig. 3e, Extended Data Table 1).

On the basis of the initial slope of the Gs-dependent increase in the rate of low-FRET state formation, we estimate the apparent Gs on rate to β2AR at around 0.03 and 0.05μM s⁻¹ for clenbuterol and adrenaline, respectively (Fig. 3e, Extended Data Table 1). These rates are orders of magnitude slower than expected for the binding of large entities18. They are, however, similar to bioluminescence resonance energy transfer measurements of β2AR-mediated Gα activation in living cells (2–3 s⁻¹)19, in which Gα lipidation localizes the heterotrimer to cellular membranes. They are also consistent with the observation that rate-limiting conformational changes within the rhodopsin–Gα complex precede GTP loading, although Gα activation occurs at a faster rate20. Hence, either initial interactions between Gs and β2AR preceding low-FRET state formation are highly transient (<100 ms) or do not immediately precipitate a FRET change. We therefore hypothesized that Gs coupling may be rate-limited by one or more ligand-dependent conformational processes that occur within a high-FRET β2AR–Gs complex.

Ligand effects on β2AR–Gs stability

To examine the stability of apyrase-treated β2AR–Gs complexes in the presence of partial and full agonists, we measured the dissociation rate of β2AR from immobilized Gs heterotrimers (Extended Data Fig. 6a). Under low illumination intensity, where photobleaching was negligible, immobilized β2AR–Gs complexes exhibited lifetimes of 5–10 min (Extended Data Table 2). Notably, these lifetimes decreased by around 20–100 fold in the presence of physiological concentrations of GDP (30μM) or GTP (100μM)21 (Extended Data Table 2), to become similar to those observed in cell-based studies of β2AR–Gs19 and β1AR–Gs complexes22. Hence, β2AR–Gs complexes generally persist for multiple seconds in the presence of nucleotide and dissociate approximately twofold faster in the presence of GTP compared to GDP. These observations are consistent with specific interactions between the receptor and nucleotide-bound G protein and suggest nucleotide-specific dissociation pathways.

To track changes in FRET efficiency within the nucleotide-depleted β2AR–Gs complex upon GDP or GTP nucleotide binding, we performed analogous experiments at high spatial and temporal resolution (Fig. 4, Extended Data Fig. 6a, e, f). In contrast to the relatively slow rates of complex dissociation, the rapid introduction (100 ms mixing time) of either GDP (30μM) or GTP (100μM) gave rise to an immediate increase, or broadening, of the low-FRET state (ΔFRET ≈ 0.05–0.10) (Fig. 4, Extended Data Fig. 6e, f). These data indicate that nucleotides bind the nucleotide-free β2AR–Gs complex at, or near, the diffusion limit, and that binding results in structural and/or dynamic changes in TM6 that initiate its return towards the helix bundle. Notably, these rapid changes were followed by the formation of a relatively long-lived, predominantly high-FRET configuration from which reversible transitions to lower-FRET states occurred with frequencies and durations that were efficacy dependent (Fig. 4, Extended Data Fig. 6e, f).

The addition of GTP triggered transitions to high-FRET that were more rapid and complete than observed for GDP, and both the rate and efficiency of high-FRET state formation were greater for full agonists than for partial agonists (Fig. 4b, d, Extended Data Fig. 6f). These findings provide direct evidence for nucleotide-specific dissociation pathways, and suggest that β2AR–Gs(GNP) complexes can access multiple conformations distinct from the nucleotide-free state observed crystallographically23. The observed persistence of high-FRET, GDP- and GTP-loaded configurations indicates the existence
of relatively long-lived, βAR–Gs(GDP) complexes during initial nucleotide addition. While the absolute rates we observe may be influenced by interactions between the relatively stable, nucleotide-free state. These findings may help to distinguish between nucleotide-free and GDP-bound βAR complexes, with a distinct mode of interaction between TM6 and the β-state (approximately 0.5) reflecting a GDP-bound βAR–Gs complex with a different mode of interaction between TM6 and the α5-helix that is relatively short lived (Fig. 4c). Because the two lower-FRET states can be seen clearly only in pre-steady-state experiments, we analysed our steady-state data as a two-state system in which states (1) and (3) are collapsed into a single, broadly defined (0.4–0.5) low-FRET state.

To learn about the rate-limiting features of βAR–Gs complex formation, we examined the rates of low- and high-FRET state formation (k_high→low and k_low→high, respectively) over a range of GDP concentrations. As expected for binding of a GDP-bound Gs heterotrimer, k_high→low was largely independent of GDP concentration for all agonists (Extended Data Fig. 8a). By contrast, k_low→high increased with GDP and plateaued at concentrations above 20 μM (Fig. 5a). Consistent with TM6 dynamics occurring within the βAR–Gs complex, the maximum rates exiting low FRET were approximately 5–15-fold more rapid than the apparent Gs dissociation rate (Extended Data Table 2). The rank order of the low- to high-FRET state transition was: clenbuterol, salbutamol, salmeterol, BI-167107, isoproterenol and adrenaline. Given that GDP binding to the nucleotide-free βAR–Gs complex is rapid (Fig. 4a, c, Extended Data Fig. 6e), we conclude that the transition out of low-FRET states into high-FRET states is rate-limited by one or more ligand-dependent processes within the βAR–Gs complex.

As low FRET includes both nucleotide-free and GDP-bound complexes (Fig. 4a, c), we speculated that the slower rates of return to high FRET observed for full agonists (Fig. 5a) may reflect higher proportions of the relatively stable nucleotide-free state. We therefore undertook an evaluation of differences in the proportion of nucleotide-free βAR–Gs complexes in the presence of distinct agonists using the experimentally observed mean value of the low-FRET state as a function of GDP concentration (Fig. 5b, c). This analysis revealed that the low-FRET state values observed at saturating GDP concentration (100 μM) were considerably lower for full agonists compared to partial agonists, more closely approximating the FRET value (0.4–0.5) observed for the nucleotide-free βAR–Gs complex (Extended Data Fig. 8c, d). Consistent with GDP binding promoting a return to the high-FRET state, increasing GDP concentrations increased the mean values of the low-FRET states, while decreasing their time-averaged occupancies (Fig. 5b, c). The concentration dependence of these effects revealed that full agonists exhibited EC50 values that were approximately 2–3-fold higher than for partial agonists (Fig. 5d, Extended Data Fig. 8e). These data suggest that βAR–Gs complexes exhibit higher affinity for GDP when bound to partial agonists than when bound to full agonists. They also support the notion that low-FRET states represent a mixture of nucleotide-free and GDP-bound βAR–Gs configurations, with complexes activated by full agonists spend more time on average in the relatively stable, nucleotide-free state. These findings may help to explain why adrenaline promotes a greater extent of [3H]GTP release from βAR compared to salbutamol27, despite both agonists promoting low-FRET states at similar rates (Fig. 3e). As the rates of GDP binding to nucleotide-free βAR–Gs complexes are rapid, and appear indistinguishable at the present time resolution (Fig. 4a, c), we conclude that more efficacious agonists increase the probability of GDP release and thus the likelihood that nucleotide-free states are achieved.

To test this model directly, we performed analogous GTP titrations in the presence of a fixed, saturating GDP concentration (30 μM) (Extended Data Fig. 7b, c). As anticipated, the transition rate from low-to-high FRET (k_low→high) was in all cases specifically increased at even the lowest GTP concentrations tested (100 nM) (Fig. 5e, Extended Data Fig. 8b). While the absolute values of k_low→high were greater for partial agonists in the presence of GTP (Fig. 5e), the fold increase in rate, and hence the magnitude reduction in low-FRET state lifetime, correlated with ligand efficacy in the order: adrenaline, isoproterenol, BI-167107, salmeterol, salbutamol and clenbuterol (Fig. 5f, Extended Data Fig. 8h). Taken together with the rapid rates of GDP and GTP binding to nucleotide-free βAR–Gs complexes (Fig. 4), we conclude that...
that the relatively short-lived, active-like, low-FRET $\beta_2$AR conformations observed in the presence of partial agonists (Fig. 5a, e, f) predominantly reflect failed attempts at nucleotide release, and that full agonists more efficiently promote relatively long-lived, nucleotide-free configurations (Extended Data Fig. 8f, g). Hence, ligands with greater efficacy preferentially promote GDP release, and under competitive conditions, rapid and efficient GTP loading to the subpopulation of nucleotide-free complexes. The more rapid return of $\beta_2$AR–Gs(GTP) complexes to high-FRET states after GTP loading argues that the terminal phosphate of GTP lowers the barrier for the rate-limiting conformational transition that enables the return of TM6 to its position adjacent to the helix bundle. This distinction may reflect GTP-specific effects on G$_a$ heterotrimer stability.

**Discussion**

We have examined ligand-activated G$_a$ binding, nucleotide exchange and G$_a$ release, from the perspective of time-dependent changes in $\beta_2$AR conformation. The results illuminate the established concept of ligand efficacy in terms of a specific kinetic framework for the activation pathway (Fig. 6a). Quantifying the ligand-dependence of both the rate and the efficiency of G$_a$ coupling in the presence of physiologically relevant GDP concentrations revealed that the process is achieved by rate-limiting conformational processes intrinsic to the $\beta_2$AR–G$_a$ complex (Fig. 3e). Although the nature of the interactions preceding excursions to low-FRET, active-like conformations are not presently known, the rates evidenced at saturating G$_a$ concentration (Fig. 3e) suggest ligand-specific effects on the probability that G$_a$, productively engages, and forms stabilizing conformational states within the G-protein

**Figure 5 | Nucleotide exchange efficiency is ligand dependent.** a. Transition rates from low- to high-FRET states ($k_{\text{low} \rightarrow \text{high}}$) with agonists, G$_a$, and increasing GDP concentrations. b, c. Low-FRET state distributions with increasing GDP in G$_a$ and clenbuterol (b) or adrenaline (c). Vertical lines indicate the mean low-FRET value observed without GDP (black; L) and the mean high-FRET value at 100 $\mu$M GDP (purple; H). d. Apparent EC$_{50}$ values for GDP binding to the $\beta_2$AR–G$_a$ complex with different agonists. e. Transition rates from low- to high-FRET states ($k_{\text{low} \rightarrow \text{high}}$) with agonists, G$_a$, GDP and increasing GTP concentrations. f. Histograms of low-FRET state lifetimes ($\tau$) for each agonist with G$_a$ and saturating GTP (light grey) or saturating GDP and GTP (dark grey). Error bars represent s.d. (two replicates) except in d, in which they represent s.e.m.

**Figure 6 | Proposed kinetic framework underlying $\beta_2$AR–G$_a$ coupling and nucleotide exchange.** a. Schematic of $\beta_2$AR TM6 conformational states within the G-protein activation cycle. (Note the low-FRET $\beta_2$AR–G$_a$(GTP) complex was not experimentally observed but is inferred.) b. G$_a$ α5 helix disrupting helix-proximal $\beta$6α5 loop interactions with GTP. c. Ranking of agonist molecular efficacies (ε) relative to adrenaline in terms of the effective rate of generating G$_a$(GTP) from G$_a$(GDP) (Supplementary Methods). d. Ligand efficacy-based cAMP measurements in living cells (Supplementary Methods; error bars denote s.e.m., three replicates). $E_{\text{max}}$, maximum agonist response.
GTP binding rates and/or affinities, which could not be accurately quantified.

In addition to providing quantitative insights into ligand efficacy, the present smFRET studies shed light on $\beta_2$AR–G protein conformations that are structurally distinct from the nucleotide-free complex (Fig. 1a). While GDP- and GTP-bound complexes may be too unstable for crystallographic study, in-depth characterizations of these states are expected to provide important insights into both G-protein coupling rates and specificities. Quantitative single-molecule imaging investigations will be crucial in such efforts, as well as for delineating distinct ligand-dependent GPCR signalling pathways.

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.

Data Availability The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Received 17 August 2016; accepted 7 April 2017.

Published online 7 June 2017.

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Supplementary Information is available in the online version of the paper.

Acknowledgements We thank M. Howarth for the gift of trans-divalent streptavidin, and C. Stern in the laboratory of J. Chodera for constructing the CHARMM-consistent parameters for the dyes used in the molecular dynamics simulations. Computational resources are gratefully acknowledged: an XSEDE allocation at the Texas Advanced Computing Center at the University of Texas at Austin (Stampede supercomputer, project TG MCB120008), support from resources at the Oak Ridge Leadership Computing Facility (ALLC allocation BIP109) at the Oak Ridge National Laboratory that is supported by the Office of Science of the US Department of Energy under contract no. DE-AC05-00OR22725; and the resources of the David A. Cofrin Center for Biomedical Information in the HRH Prince Alwaleed Bin Talib Bin Abdulaziz Alsaud Institute for Computational Biomedicine at Weil Cornell Medicine. This work was supported in part by National Institutes of Health (NIH) grants GM098859 (S.C.B.), R21DA035485 (J.A.J.), S.C.B. and G.O.G., RO1DK054224-13 (J.A.J.), R01GM083118 (D.H.), R01NS028471 (B.K.K.), and U54GM087519 (H.W. and J.M.P.-A.), the German Academic Exchange Service (DAAD) (D.H.), the American Heart Association Postdoctoral fellowship (15POST22700200) (M.M.), and the Novo Nordisk Foundation Center for Basic Metabolic Research (M.H.).

Author Contributions G.G.G., M.M., D.H., B.K.K. and S.C.B. designed single-molecule experiments. G.G.G. labelled receptor and performed all single-molecule experiments. G.G.G. analysed single-molecule data, with support from D.S.T. M.J. and D.S.T. developed the imaging and analysis platform. M.M. expressed, purified and characterized receptor constructs. D.H. expressed, purified and bioquantified G protein-coupled receptors. D.H. expressed, purified and biotinylated G protein-coupled receptors. B.K.K. and S.C.B. provided overall project supervision.

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Reviewer Information Nature thanks M. Lohse and the other anonymous reviewer(s) for their contribution to the peer review of this work.
Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Ligand-binding properties of β2Δ6-148C/266C. a, [3H]dihydroalprenolol (DHA) saturation binding on purified β2AR, comparing wild type (WT; blue squares) versus unlabelled (orange circles) and labelled (green triangles) β2Δ6-148C/266C. Affinities (Kd values) are shown in the table. Non-specific binding controls are shown as corresponding open symbols. Error bars denote the s.e.m. from triplicate measurements. b, [3H]DHA–isoproterenol competition binding on purified β2AR, comparing wild type (blue circles) versus unlabelled (orange squares) and labelled (green triangles) β2Δ6-148C/266C. Affinities (Ki values) are reported in the table. Error bars represent the s.e.m. from triplicate measurements. c, Dose–response curves of the BRET-based cAMP biosensor CAMYEL with wild-type β2AR (black triangles) and the mutants β2Δ6 (blue squares) and β2Δ6-148C/266C (red circles). Data from untransfected cells are shown in green triangles. Data from five independent experiments were normalized to the maximal isoproterenol response by wild-type β2AR in each experiment and globally fit to the entire dataset, with the error bars representing the s.e.m., as reported in the table. d, Skeletal structures of ligands used in the current study. e, [3H]DHA competition binding on unlabelled β2Δ6-148C/266C for ligands used in the current study, except for carazolol and BI-167107, for which the ultra-high affinities reported (32 pM and 84 pM, respectively) would not allow us to determine them accurately in our assay. Instead, we used concentrations of 1 μM for both in all our measurements. The calculated Ki values are shown on the table.
Extended Data Figure 2 | Fluorophore structures and properties.

**a, b**, Skeletal structures of the modified Cy3B* donor (a) and Cy7* acceptor (b) fluorophores. **c, d**, Normalized donor fluorophore emission (Cy3: green; Cy3B*: dark green) and acceptor fluorophore absorbance (Cy5: red; Cy7*: dark magenta) spectra for Cy3/Cy5 (c) and Cy3B*/Cy7* (d) FRET pairs. The spectral overlap integral (shaded region) was calculated and used to determine the Förster distance ($R_0$) values for each pair. **e**, Inter-dye FRET efficiencies of the Cy3/Cy5 (black) and Cy3B*/Cy7* (blue) donor and acceptor fluorophore pairs as a function of inter-dye distances calculated based on $R_0$ values. **f**, Bulk anisotropy measurements on Cy3B*-labelled $\beta_2\Delta6-148C/266C$. © 2017 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | smFRET experimental controls. a, Site-specific labelling. SDS–PAGE gels under green (540 nm) or near infrared (740 nm) illumination for fluorescence visualization of Cy3B* or Cy7* labelling of β2Δ6 and β2Δ6-148C/266C. Coomassie-stained gel image is shown as a gel-loading control. Digestion with factor Xa protease and deglycosylation with PNGase F leads to separation of the 148C and 266C labelling sites on generated N-terminal and C-terminal fragments, respectively. For gel source data, see Supplementary Fig. 1. b, Quantification of Cy3B*/Cy7*-labelling specificity of full-length β2Δ6-148C/266C. Data are normalized to β2Δ6-148C/266C labelling. c, Specificity of streptavidin-mediated receptor immobilization. Frame capture from immobilization movies showing labelled β2Δ6-148C/266C on streptavidin-free (−SA) or streptavidin-coated (+SA) surfaces. Bar graph shows the number of immobilized, labelled β2AR in these conditions. Error bars represent the s.d. from two replicates. d, Schematic of labelled β2AR immobilization via biotinylated alprenolol (alp-biotin). e, FRET population contour plot and histogram for alp-biotin-immobilized receptor shows correspondence with the FRET population distribution of biotin-M1-Fab-immobilized, alprenolol-bound, labelled β2AR (Fig. 2b). Histogram error bars represent the s.d. from four replicates, with n total molecules analysed. f, FRET population contour plots (top) and histograms (bottom) for adrenaline titration on biotin-M1-Fab-immobilized, labelled β2Δ6-148C/266C (Fig. 2c). Dashed lines (blue) highlight the mean FRET values for the lowest (2 nM; top dashed line) and highest (200 μM; bottom dashed line) adrenaline concentrations tested. Histogram error bars represent the s.d. from three replicates with n total molecules analysed. The scale bar on the right indicates relative populations for the contour plots.
Extended Data Figure 4 | TM6 motions within ligand-bound β₂AR.

a, Sample fluorescence (green for Cy3B*; magenta for Cy7*) and FRET (blue) time traces for biotin-M1-Fab-immobilized, labelled β₂Δ6-148C/266C imaged in the absence and presence of saturating ligands. b, Same as Fig. 2b but with the full FRET efficiency range (0–1) shown for the population histograms (bottom). c, Plots showing the mean cross correlation values of donor and acceptor fluorescence as a function of lag time for the ensemble of individual fluorescence traces obtained from experiments shown in Fig. 2b. d, Plots showing the mean cross correlation values of donor and acceptor fluorescence as a function of lag time for an ensemble of simulated fluorescence trajectories rapidly fluctuating between high (0.75) and intermediate (0.55) FRET values with varying low- to high-FRET transition rate constants (coloured lines), where the high- to low-FRET transition rate is held constant at 100 s⁻¹ (Supplementary Methods). e, FRET distributions of the simulated data, as described in d.
Extended Data Figure 5 | All-atom molecular dynamics simulations of the Cy3B*/Cy7*-labelled β2AR in a detergent micelle. a, Time evolution of the distance between the dyes. Time dependence of the distances between the midpoints of the dyes along the simulation trajectories is shown for the β2AR–carazolol (grey) and β2AR–BI-167107/Gs (black) systems. The distributions are displayed as histograms on the right; grey bars: β2AR–carazolol; clear bars: β2AR–BI-167107/Gs. The estimated inter-dye distances derived from the experimental mean FRET values (Figs 2b, 3b; Extended Data Fig. 2e) are indicated by solid lines topped with circles (β2AR–carazolol: red; β2AR–BI-167107/Gs: blue). b, Time evolution of the distance between Cα carbons at the labelling site. Cα–Cα distances for β2AR–carazolol: red and β2AR–BI-167107/Gs: blue. c, The simulated dye-tethered β2AR–BI-167107/Gs system embedded in a n-dodecyl-β-d-maltoside (DDM) micelle (grey sticks). β2AR is rendered in grey, with TM6 and the agonist (BI-167107) highlighted in blue. The Gs protein is rendered in wheat colour surrounded by its molecular surface to indicate the excluded volume for dye movements. The Cy3B* and Cy7* dyes are coloured green and magenta, respectively. Water molecules, ions and detergent molecules distant from the β2AR structure are omitted. d, Positions explored by the midpoints of the dyes during the simulations are shown as clusters of dots in the context of the β2AR–carazolol (transparent red dots) and β2AR–BI-167107/Gs (transparent blue dots) complexes; the centre of mass of each collection of dots is indicated by a solid sphere. Cα carbons for labelled positions 148 and 266 are shown as magenta and green spheres, respectively.
Extended Data Figure 6 | smFRET imaging of biotin-Gs-immobilized, labelled β2AR. a, Schematic of labelled β2AR immobilization via biotinylated Gs heterotrimer. b, c, Representative fluorescence (green for Cy3B*; magenta for Cy7*) and FRET (blue) time traces for biotin-Gs-immobilized, labelled β2Δ6-148C/266C imaged in the presence of clenbuterol (b) or adrenaline (c) in nucleotide-free conditions (apyrase-treated). d, FRET population contour plots (top) and histograms (bottom) for biotin-Gs-immobilized β2Δ6-148C/266C imaged in the presence of partial and full agonists in nucleotide-free conditions. The dashed line indicates the invariant mean FRET value (~0.38) for all agonists tested. Histogram error bars represent the s.d. from three replicates, with n total molecules analysed. e, f, FRET population contour plots of biotin-Gs-immobilized β2Δ6-148C/266C in the presence of agonists exhibiting FRET transitions upon rapid addition (arrow) of 30μM GDP (e) or 100μM GTP (f). Scale bar on the right indicates the relative population for the contour plots.
Extended Data Figure 7 | Ligand-dependent TM6 dynamics in the presence of Gs, GDP and GTP. a, Sample fluorescence and FRET time traces for biotin-M1-Fab-immobilized, labelled β2Δ6-148C/266C imaged in the absence and presence of saturating ligands plus 8 µM Gs in nucleotide-free (after apyrase treatment) conditions. b, FRET population contour plots for biotin-M1-Fab-immobilized, labelled β2Δ6-148C/266C imaged in the presence of the agonists clenbuterol (b) or adrenaline (c), 8 µM Gs, and increasing concentrations of GDP (Fig. 5a) or GTP in the presence of saturating GDP (30 µM) (Fig. 5e), with n indicating the total number of molecules analysed from two replicates. Nucleotide-free (0 µM GDP; from Fig. 3b) and ligand-only (from Fig. 2b) conditions are included as references. Scale bar on the right indicates the relative population for the contour plots.
Extended Data Figure 8 | Steady-state dynamics within β2AR related to nucleotide exchange. a, b, Transition rates from high- to low-FRET states (k_{high→low}) of labelled β2AR with different agonists, saturating G_s (8 μM) and increasing GDP concentrations (2–100 μM) (a) or increasing GTP concentrations (0–100 μM) in 30 μM GDP (b). c, Low-FRET state distributions for labelled β2AR with different agonists in 100 μM GDP and saturating G_s showing their overlap with the distribution in the presence of adrenaline and saturating G_s in nucleotide-free conditions (apyrase) (Fig. 3b). The dashed line shows the mean FRET value for apyrase. d, Mean low FRET values from c. e, The percentage change in the area of the low-FRET state population distributions for clenbuterol (black squares) and adrenaline (dark yellow triangles) (as shown in Fig. 5b and c, respectively) was plotted with increasing GDP concentrations (0–100 μM) and fitted to a single exponential decay function to derive the GDP EC_{50} value. f, g, Sample FRET traces (blue line) of labelled β2AR in the presence of clenbuterol (f) or adrenaline (g) plus 100 μM GDP and saturating G_s. Dashed lines indicate each ligand’s corresponding mean high FRET value (black), mean low FRET value (light green) and mean FRET value in nucleotide-free conditions (dark green). h, The ratio of the low-FRET state lifetime of β2AR in the presence of saturating G_s and GDP (τ_{GDP}) over the low-FRET state lifetime in saturating GTP plus 30 μM GDP (τ_{GDP+GTP}) (Fig. 5f) is shown for different agonists. All error bars represent s.d. from two replicates.
Extended Data Table 1 | Apparent on rate and transition rates from high- to low-FRET states

|      | apparent $k_{on}$ (µM$^{-1}$s$^{-1}$) | $k_{high\rightarrow low}$ (s$^{-1}$) |
|------|----------------------------------------|-----------------------------------|
| ALP  | <<0.02                                 | -                                 |
| CLEN | 0.03                                   | 0.4                               |
| SALM | 0.04                                   | 0.6                               |
| SALB | 0.04                                   | 0.7                               |
| BI   | 0.04                                   | 0.7                               |
| ADR  | 0.05                                   | 0.7                               |
| ISO  | 0.04                                   | 1.1                               |

$k_{on}$ and $k_{high\rightarrow low}$ values were calculated from the data presented in Fig. 3e.
Extended Data Table 2 | Lifetimes of biotin-G\(_\alpha\)-immobilized and biotin-M1-Fab-immobilized \(\beta_2\)AR

| Ligand | Lifetime (min) | GNP-free (min) | +GDP (s) | +GTP (s) |
|--------|----------------|----------------|----------|----------|
| CLEN   | 14.4 ± 2.1     | 8.3 ± 1.3      | 9.8 ± 1.6| 6.8 ± 0.5|
| SALB   | 18.1 ± 8.4     | 5.0 ± 0.4      | 8.2 ± 0.9| 6.4 ± 0.1|
| SALM   | 10.8 ± 4.1     | 3.5 ± 1.6      | 9.0 ± 0.4| 6.6 ± 0.2|
| BI     | 11.0 ± 5.5     | 8.0 ± 3.2      | 9.4 ± 0.6| 6.2 ± 0.1|
| ISO    | 12.7 ± 3.1     | 6.0 ± 5.2      | 9.8 ± 1.3| 5.8 ± 0.1|
| ADR    | 13.2 ± 3.4     | 11.2 ± 5.1     | 11.8 ± 1.2| 6.4 ± 0.1|

Lifetime values are shown for biotin-G\(_\alpha\)-immobilized \(\beta_2\)AR in the nucleotide-free state (GNP-free) and after the addition of GDP or GTP, and for biotin-M1-Fab-immobilized \(\beta_2\)AR in the absence of \(\alpha_\text{a}\). Data are mean ± s.d. from two replicates.