Metastable GPCR dimers trigger the basal signal by recruiting G-proteins

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G-protein-coupled receptors (GPCRs) constitute the largest family of integral membrane proteins in the human genome and are responsible for various important signaling pathways for vision, olfaction, gustation, emotion, cell migration, etc. A distinct feature of the GPCR-family proteins is that many GPCRs, including the prototypical GPCR, β 2-adrenergic receptor (β 2AR), elicit low levels of basal constitutive signals without agonist stimulation, which function in normal development and various diseases¹⁻³. However, how the basal signals are induced is hardly known. Another general distinctive feature of GPCRs is to form metastable homo-dimers, with lifetimes on the order of 0.1 s, even in the resting state. Here, our single-molecule-based quantification⁴ determined the dissociation constant of β 2AR homo-dimers in the PM (1.6 ± 0.29 copies/ μm^2) and their lifetimes (83.2 ± 6.4 ms), and furthermore found that, in the resting state, trimeric G-proteins were recruited to both β 2AR monomers and homo-dimers. Importantly, inverse agonists, which suppress the GPCR's basal constitutive activity, specifically blocked the G-protein recruitment to GPCR homo-dimers, without affecting that to monomers. These results indicate that the G-proteins recruited to transient GPCR homo-dimers are responsible for inducing their basic constitutive signals. These results suggest novel drug development strategies to enhance or suppress GPCR homo-dimer formation.

β 2AR forms metastable homo-dimers

Single-molecule imaging-tracking revealed that β 2AR (tagged with ACP at the N-terminus and labelled with ATTO594; **Extended Data Fig. 1**) undergoes thermal diffusion in the plasma membrane (PM), and often forms transient homo-dimers, undergoing rapid interconversions between monomers and dimers continually, as found for other GPCRs (**Fig. 1a, Extended Data Fig. 2a**)⁴⁻⁷. Every time we found a β 2AR dimer, we measured the dimer duration, and after observing many dimers, we obtained the distribution of dimer durations (**Fig. 1b, Extended Data Fig. 2d**). The histogram could be fitted by a single exponential function, providing the exponential dimer lifetime of 99 ms (throughout this report, all statistical parameters, including SEM, and the

number of experiments are summarised in **Extended Data Tables 1-4**; all observations were performed at 37°C). The lifetime value obtained in this manner, termed τ_{observed} , was corrected for the photobleaching lifetime and incidental colocalisation lifetime (this method was used for evaluating all of the lifetimes investigated in the present research; see **Methods**), giving a dimer lifetime τ of 83.2 ± 6.4 ms (**Fig. 1c**). The dimer lifetime obtained at a time resolution of 4 ms (250 Hz) was 78.8 ± 10.7 ms, after corrections for photobleaching and incidental colocalisation lifetime (**Extended Data Fig. 3, Fig. 1c**), which is statistically indistinguishable from the dimer lifetime of 83.2 ms evaluated from 30-Hz observations. This result indicates that a frame rate of 30 Hz is sufficiently fast for evaluating the homo-dimer lifetime of β 2AR. Together, these results demonstrate that the β 2AR homo-dimers are indeed quite metastable, turning into monomers in a matter of 80 ms, and then quite readily forming another dimer with a different partner molecule (**Extended Data Fig. 2a**).

2D-dissociation constant for β 2AR dimers

Using single-molecule-based “super-quantification” method previously developed by us⁴, we obtained the 2-dimensional dissociation constant ($2D-K_D$) of β 2AR homo-dimers in the PM of living cells, which was 1.6 ± 0.29 copies/ μm^2 (**Fig. 2a, Extended Data Fig. 2b**; see **Methods**). This value is comparable to the $2D-K_D$ of another GPCR, formyl-peptide receptor (FPR), which was 3.6 copies/ μm^2 (in fact, this was the first determination of the $2D-K_D$ in the PM of living cells)⁴.

Under physiological conditions, β 2AR is expressed at number densities of $16 \sim 260$ copies/ μm^2 , depending on the cell type^{8,9}. From the $2D-K_D$ determined here, we conclude that $12 \sim 240$ copies/ μm^2 exist as dimers and $4 \sim 20$ copies/ μm^2 exist as monomers at any moment; *i.e.*, 75 $\sim$ 92% of β 2AR molecules exist as homo-dimers at any moment, although dimers and monomers are continually interconverting rapidly all the time (with a dimer lifetime of ~ 80 ms). In contrast, FPR is mainly expressed in neutrophils, and an average of about 6,000 copies of FPR exist in the PM of a single neutrophil¹⁰. Assuming that the neutrophil surface area is $1,300 \mu\text{m}^2$ (approximated by a sphere with a radius of $10 \mu\text{m}$, providing a number density of ~ 4.6 FPR copies/ μm^2) and based on the $2D-K_D$ of FPR (3.6 copies/ μm^2), 3,200 copies of FPR would exist as monomers and 2,800 copies

as dimers; namely, 54% of FPR molecules are dimers, which is about two-thirds of the dimer fraction of β 2AR. Note that, even under the conditions where the dimer population dominates (*i.e.*, at higher expression levels), the dimer lifetimes do not change and remain on the order of 0.1 s. Only the monomer lifetimes are shortened at higher expression levels.

Direct detection of β 2AR dimers by the BiFC method

Thus far, we have detected dimers by the colocalisation of two single molecules, which reveals the close encounters of molecules in the space scale of 220 nm, a distance much greater than the molecular scale. Therefore, in this method, the incidental approaches of two molecules were evaluated and subtracted. To ascertain the molecular-level interactions (dimers), we employed YFP-based bimolecular fluorescence complementation (BiFC; meanwhile, our attempt to detect FRET did not work, perhaps because the distance between the probes attached to two C-termini was too far for FRET)^{4,11}. In BiFC, two potentially interacting proteins are fused to the N- and C-terminal half-molecules of YFP. If the proteins of interest form dimers, then YFP may be reconstituted and emit fluorescence signals (see **Methods**). Thus, if BiFC occurs, it would lend strong support for the formation of β 2AR dimers.

To quantitatively examine BiFC, we varied the expression levels of β 2AR. With an increase in the number density of expressed molecules (determined by the signal from ATTO594 bound to ACP; the ATTO594/ACP ratio is $>0.95^{12}$), the number density of the BiFC spots of β 2AR increased, whereas the BiFC-spot number density of the monomer reference molecule, ACP-TM, increased only slightly (**Fig. 2b; Extended Data Fig. 2c**). This result indicates that β 2AR forms directly-bound dimers.

Inverse agonists reduce the β 2AR dimer affinity

Treatments with the inverse agonists ICI-118,551 (ICI) and timolol reduced the β 2AR dimer lifetime (0.74x and 0.69x, respectively; **Fig. 1c, Extended Data Fig. 2d**), whereas ICI increased the 2D- K_D of β 2AR by a factor of 2.1 (lowered the β 2AR dimer affinity; **Fig. 1c**), indicating that inverse agonists reduce the β 2AR dimer affinity. Meanwhile, the addition of the agonist isoproterenol (Iso) prolonged

the dimer lifetime significantly (1.55x) (**Fig. 1c; Extended Data Fig. 2d**). Cholesterol reportedly modulates the functions of some GPCRs¹³, but here we found that mild cholesterol depletion by methyl- β -cyclodextrin (M β CD) treatment failed to affect the β 2AR dimer lifetime (**Fig. 1c**).

Since the inverse agonists suppressed the basal constitutive GPCR signaling, and since we found that they also reduced the β 2AR dimer lifetime, we next examined the dimer lifetimes of the β 2AR mutants with high and low basal activities in the resting state (HBA and LBA mutants, representing E268A and C327R mutants, respectively^{14,15}). The HBA mutant exhibited a 1.78x longer dimer lifetime, and interestingly, after the inverse-agonist addition, it was reduced to the dimer lifetime of the wild-type receptor (**Fig. 1c; Extended Data Fig. 2d**). Meanwhile, the LBA mutant exhibited a 0.74x shorter dimer lifetime than that of the wild-type receptor, and the agonist addition did not affect it (**Fig. 1c; Extended Data Fig. 2d**).

Strikingly, the dimer lifetimes of WT- β 2AR after the addition of inverse agonists were quite comparable to those of the LBA mutant, and the dimer lifetime of WT- β 2AR after the addition of the agonist isoproterenol was comparable to that of the HBA mutant. These results suggest that the β 2AR conformations would be quite different after the binding of the inverse agonists and the agonist isoproterenol, as compared with those before their additions¹⁶, possibly resembling the conformations of the LBA and HBA mutants, respectively, and detectable as changes in the homo-dimer lifetimes.

The basal constitutive activity of β 2AR

We confirmed the basal constitutive activity of β 2AR, as well as the effects of the β 2AR agonist isoproterenol and the inverse agonists ICI-118,551 and timolol, by measuring the changes in the cytoplasmic cAMP levels in the L-cell line stably expressing β 2AR at higher levels (**Extended Data Fig. 4, Extended Data Table 4**). The addition of the agonist isoproterenol to the cells increased the cAMP level by a factor of ~ 4 , relative to the control. Meanwhile, the addition of the inverse agonists ICI-118,551 and timolol reduced the cAMP levels by factors of 1.9 and 1.3 (53 and 76% of the control), respectively, showing the presence of the basal constitutive activity of β 2AR and the effectiveness of the inverse agonists, ICI-118,551 and timolol, for strongly reducing this basal

activity.

Trimeric G proteins are recruited to both β 2AR monomers and dimers

The presence of the basal constitutive activity of β 2AR suggests that a stimulatory trimeric G-protein might be recruited to β 2AR in the resting state¹⁷. Therefore, we examined whether $G\alpha_s$ and $G\beta$ (conjugated with mGFP) are recruited to β 2AR monomers or dimers, by examining their colocalisations. Using L cells expressing both β 2AR (ATTO594-ACP- β 2AR) and $G\alpha_s$ or $G\beta$, we found that, even in resting cells, $G\alpha_s$ and $G\beta$ were recruited to both β 2AR monomers and dimers (**Fig. 3a; Supplementary Videos 1 and 2**).

The recruitment frequencies of $G\alpha_s$ and $G\beta$ to β 2AR monomers and dimers were measured and then normalised by the number densities of $G\alpha_s$ (or $G\beta\gamma$) and β 2AR protomers in the PM (normalised frequencies, with a dimension of per sec per μm^4 ; **Fig. 3b, c; Extended Data Table 3**)¹⁸. In resting cells, both $G\alpha_s$ and $G\beta$ molecules were recruited to β 2AR monomers or dimers equally well, suggesting that (non-stimulated) β 2AR monomers and/or dimers might induce intracellular Gs-dependent signals even in the resting state, which would lead to the basal constitutive activity of β 2AR.

The exponential dwell lifetimes of G proteins on β 2AR were on the order of 50 ~ 60 ms for all of the combinations of $G\alpha_s$ and $G\beta$ molecules vs β 2AR monomers and dimers (**Extended Data Fig. 5**). However, these durations are only the maximal estimates of the dwell lifetimes of $G\alpha_s$ and $G\beta$ molecules on β 2AR monomers and dimers, due to the present instrumental limitations of detecting short binding durations.

Notably, the treatment of the cells with the agonist, isoproterenol, did not change the recruitment frequencies in any combinations of $G\alpha_s$ and $G\beta$ vs β 2AR monomers and dimers, although it significantly enhanced the cytoplasmic cAMP level (**Extended Data Fig. 4, Extended Data Table 4**). This result is quite surprising, but it suggests that the isoproterenol-induced β 2AR conformation enhanced its GEF activity, rather than increasing the on-rate of G protein binding to β 2AR.

Inverse agonists specifically block G-protein recruitment to β 2AR dimers

Next, we examined the effects of the inverse agonists. The recruitment of $G\alpha_s$ and $G\beta$ to β 2AR dimers was almost completely blocked, whereas that to β 2AR monomers was unaffected (**Fig. 3b, c**). This result unequivocally shows that β 2AR dimers are responsible for the basal constitutive activity of β 2AR. Furthermore, it demonstrates that the inverse agonists work by blocking the recruitment of stimulatory G-proteins to β 2AR dimers, perhaps by inducing β 2AR conformational changes in the binding sites for G-proteins, rather than blocking the β 2AR dimerisation (although, as already described, the dimer affinity of the inverse agonist-bound β 2AR is lower, resembling the LBA mutant, and thus inverse agonists might change the GPCR conformations in two ways: lowering the GPCR dimer affinity and modulating the G-protein binding sites).

To further confirm that β 2AR dimers trigger downstream signaling without an agonist, and to detect the basal dimer signal at very low β 2AR expression levels used for single-molecule experiments (generally <1 copy/ μm^2 , which is approximately comparable to $<3,000$ copies in the PM/cell), we examined whether artificially-induced stable β 2AR dimers could elicit the downstream signal. To detect the downstream signal at very low expression levels of β 2AR, which are necessary for detecting dimers by single-molecule imaging, and also to detect the signal in a different manner, rather than observing the cAMP level again (**Extended Data Fig. 4**), we examined the intracellular Ca^{2+} mobilisation, which could occur further downstream from the cAMP amplification¹⁹. The induction of the artificially-induced stable β 2AR (conjugated to FKBP) dimers by the FKBP-A20187 dimerisation system indeed elicited Ca^{2+} mobilisation (**Extended Data Fig. 6**). This result further confirmed that β 2AR dimers could trigger the downstream signals without agonist stimulation.

Discussion

All of the class-A GPCRs examined thus far, including β 2AR examined here and by others⁵, FPR, D2 dopamine receptor, M1 muscarinic receptor, and α 1AR, form metastable dimers with lifetimes on the order of 0.1 s in the non-stimulated state⁴⁻⁷. Therefore, we propose that the dynamic equilibrium between monomers and homo-dimers is a common characteristic property of the class-A GPCRs. The homo-dimerisation of GPCRs without any noticeable amino-acid homologies suggests that,

191 through evolution, GPCRs changed almost all of their amino acids, but conserved the physical ability
192 to form transient homo-dimers. This infers that these dynamic dimers are critical for some of the
193 GPCR functions.

194 GPCRs are distinct among other receptors, in that, under non-stimulated conditions, GPCRs
195 maintain the low basal constitutive activity, which can be inhibited by inverse agonists. The basal
196 activities of GPCRs are implied in normal development and various diseases¹⁻³. Therefore, we
197 examined the possibility that the function of the GPCR dimers is to induce the basal constitutive
198 activity in resting cells (without agonists), and obtained three unexpected, surprising findings. First,
199 both $\beta 2AR$ monomers and dimers continually recruit G proteins, one molecule after another, even in
200 the resting state. Second, the G-protein recruitment frequencies in the resting state are about the
201 same in the presence of the agonist. Third, more importantly, the inverse agonists specifically and
202 almost completely blocked the recruitment of $G\alpha s$ and $G\beta$ to $\beta 2AR$ dimers, without affecting their
203 recruitment frequencies to $\beta 2AR$ monomers (**Fig. 3b, c**). The third result unequivocally shows that
204 the G proteins bound to $\beta 2AR$ dimers, and not monomers, in the resting state are responsible for
205 triggering the $\beta 2AR$'s basal constitutive activity, which is sensitive to inverse agonism. It further
206 suggests that the binding of the inverse agonists to $\beta 2AR$ induces conformational changes that
207 decrease the $\beta 2AR$ dimer affinities for G proteins (**Fig. 2a**) and at the same time, hide the binding
208 site(s) for G proteins in the dimer conformations.

209 Although the function of the GPCR dimers is to maintain the basal constitutive activity, it is
210 critically important to realise that the GPCR dimers are metastable with lifetimes on the order of 0.1
211 s. Therefore, the GPCR dimers are dispersing continually, forming new dimers with other partner
212 molecules all the time. In cells, this allows the basal constitutive activities of GPCRs to be maintained
213 at certain levels, and yet the levels of the basal GPCR signals (the average number density of GPCR
214 dimers at any time) are regulated globally, by modulating the GPCR number density by varying the
215 expression levels of the GPCR, and locally, by temporarily and spatially varying the actin-based
216 membrane skeleton and the membrane curvature^{5,20-22}. In addition, the metastable GPCR dimers
217 might play important roles in the activation of G proteins after the agonist addition.

218 The present results suggest that the major functions of some of the orphan GPCRs might be

219 performed by the basal activities induced by their homo-dimers, as the GPCR family includes many
 220 orphan receptors^{3,23}. Based on the results described in this report, we strongly suggest the pursuit of
 221 drug development strategies that emphasise the generation of more inverse agonists for various
 222 GPCRs, as well as drugs that enhance or suppress GPCR homo-dimer formation.

223

224 **Online content**

225 Any methods, additional references, Nature Research reporting summaries, source data, extended
 226 data, supplementary information, acknowledgements, peer review information; details of author
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Author contributions

R.S.K. performed the single fluorescent-molecule tracking experiments and biochemical experiments. R.S.K., T.K.F., and A.K. developed and built the single-molecule imaging station. T.K.F. developed the analysis software. R.S.K. and A.K. conceived and formulated the project, evaluated and discussed the data, and wrote the manuscript, and all authors participated in revising the manuscript.

259 **Methods**

260 **Cell culture, cDNA construction and expression in L cells, and treatment of cells** 261 **with agonists and inverse agonists.**

262 L cells (a kind gift from H. Fujisawa of Nagoya University), which do not express $\beta 2AR^{24}$,
263 were confirmed to be free of mycoplasma contamination using MycoAlert (Lonza), and were
264 routinely cultured in Dulbecco's Minimum Essential Medium (DMEM; Sigma-Aldrich)
265 supplemented with 10% (v/v) FBS (Sigma-Aldrich), 100 units/ml penicillin (Wako), and 0.1
266 mg/ml streptomycin (Wako). Human $\beta 2AR$ was fused at its N-terminus to the ACP- or
267 Halo7-tag protein (NEB and Promega, respectively) at the cDNA level, and cloned into the
268 expression plasmid pEGFP-N1 (Clontech) after the removal of the cDNA region encoding
269 EGFP. The ACP- and Halo7-tag proteins do not induce dimerisation^{11,25}. For the genetic
270 conjugation of mGFP to $G\alpha s$, the mGFP cDNA was inserted between the region encoding the
271 71st and 82nd amino acids of mouse $G\alpha s$ (Origene) in the pcDNA3.1 (+) vector
272 (Invitrogen)²⁶, and for that to $G\alpha$, mGFP was fused to the N terminus of bovine $G\alpha 1$ (a kind
273 gift from T. Haga of Gakushuin University)²⁷ in the pEGFP-C1 vector (Clontech), after the
274 removal of the cDNA region encoding EGFP. The details of the constructed cDNAs are shown
275 in **Extended Data Fig. 1**. The L cells were transfected with these cDNAs using
276 LipofectAMINE LTX (Life Technologies), according to the manufacturer's recommendations.
277 Transfected cells were seeded in glass-base dishes (35 mm ϕ with a glass window of 12 mm ϕ ,
278 0.12~0.17-mm-thick glass; Iwaki), and cultured in Ham's F12 medium (Sigma-Aldrich),
279 supplemented with 10 % (v/v) FBS (Sigma-Aldrich), 100 units/ml penicillin (Wako), and 0.1
280 mg/ml streptomycin (Wako), for 24~48 h before microscope observations. The F12 medium
281 was employed because it has a lower fluorescence background as compared with DMEM,
282 which was used for routine culturing.

283 The agonist, isoproterenol (Sigma-Aldric), and the inverse agonists, ICI-118,551
284 (Tocris) and timolol (Wako), were dissolved in Hank's balanced salt solution (Nissui)
285 buffered with 2 mM piperazine-N,N'-bis(ethanesulfonic acid) (PIPES, Dojindo) at pH 7.4

(P-HBSS), at concentrations of 20, 200, and 200 μM , respectively. For the additions to the cells, 1 ml solutions of these reagents were added to the cells in 1 ml of the Ham's F12 medium described above, providing the final concentrations of 10 μM isoproterenol, 100 μM ICI-118,551, and 100 μM timolol.

291 **Labelling of β2AR with the fluorescent dye ATTO594**

ATTO594-Coenzyme A was custom-synthesised by Shinsei Kagaku, by conjugating ATTO594-maleimide (ATTO-TECH) to Coenzyme A-SH (New England Biolabs), followed by HPLC purification. The chemical structure of ATTO594-Coenzyme A was confirmed by liquid chromatography-mass spectrometry (LC-MS; LCMS-2010A EV, Shimadzu). The ACP-tagged β2AR expressed in the PM of the L cells was labelled with ATTO594, by incubating the cells with 2 μM ATTO594-Coenzyme A and 1 μM phosphopantetheine transferase (New England Biolabs) in Ham's F12 medium without FBS at $\sim 25^\circ\text{C}$ for 20 min. These conditions have been known to achieve $\sim 95\%$ labelling of ACP²⁵. The cells were washed with P-HBSS three times, and then subjected to microscope observations. The cells exhibiting the presence of typically ~ 1 ATTO594-ACP- β2AR spot/ μm^2 in the PM were used for single-molecule imaging experiments.

304 **Single fluorescent-molecule video imaging of live cells**

The fluorescently-labelled ACP- β2AR expressed in the bottom PM was observed at the level of single molecules at 37°C , using home-built objective-lens-type total internal reflection fluorescence microscopes based on Olympus IX-70, Olympus IX-81, or Nikon TE-2000 inverted microscopes^{4,28-30}, with a 100x objective lens, as optimised for the present research. Fluorescence images were projected onto a two-stage microchannel plate intensifier (C8600-03, Hamamatsu Photonics), coupled to an electron bombardment CCD camera (C7190-23; Hamamatsu Photonics) or an sCMOS camera (ORCA-Flash 4.0; Hamamatsu Photonics) operated at 30 Hz. For observations at a frame rate of 250 Hz, a CMOS camera (FASTCAM 1024PCI-II, Photron) was employed³¹. Fluorescent spots were identified by using

an in-house computer program, as described previously⁴. The positions (x and y coordinates) of all of the observed single fluorescent-molecules were determined by a computer program that employed the method developed previously^{32,33}. For the simultaneous dual-color single-molecule imaging, the images were spectrally split into two emission arms, each containing the same image intensifier and camera.

To maintain the cells at 37°C during the microscope observations, the entire microscope, except for the two detection arms and the excitation arm, was placed in a home-built microscope environment chamber made with thermo-insulating transparent vinyl sheets, and three heating circulators (SKH0-112-OT, Kokensya) were appropriately placed in the chamber to supply warmed air with slow circulation^{30,34,35}. For further stabilisation, a plate heater (INUG2, Tokai Hit) was mounted on the microscope stage. The temperature of the surrounding air just above the stage was continually monitored during the observations with a digital thermometer (DT-312, Intermedical).

327

328 **Detection of colocalisation-codiffusion, and evaluations of β 2AR dimer lifetimes**

329 **and two-dimensional dissociation constants of β 2AR dimers**

The number densities of β 2AR molecules existing as (true) dimers and the number density of the expressed β 2AR molecules were determined by single-molecule imaging of ATTO594-ACP- β 2AR⁴. Colocalisations of two fluorescent spots (molecules) of the same color (the same ATTO594 probe) or those of two different colors (YFP for BiFC and ATTO594) were detected when the two fluorescent molecules became localised within 220 nm of each other^{4,30}. The number densities of incidental colocalisations at given molecular densities were subtracted⁴. The two-dimensional dissociation constant (2D- K_D) of β 2AR in the PM was determined by using the method previously developed by us⁴.

To directly detect the actual binding of two β 2AR molecules, forming homo-dimers, we employed the bimolecular fluorescence complementation (BiFC) method^{4,36}. The details of the BiFC method are described in the next subsection.

To determine the lifetime of β 2AR dimers using single color observations (with ATTO594-ACP- β 2AR), the duration of each colocalisation-codiffusion event was measured^{4,11,29}, and after the durations of many events were determined, histograms showing the distribution of colocalisation durations were obtained. All of the histograms obtained from the image sequences obtained at a time resolution of 33 ms could be fitted by single exponential functions, which provided decay time constants (lifetime; τ_{observed}). τ_{observed} was then corrected for the photobleaching lifetime of the fluorescent probe ATTO594 ($\tau_{\text{bleach}} = 7.21 \pm 0.81$ s for 30 Hz [$n = 300$] and 0.812 ± 0.041 s for 250 Hz [$n = 539$]), to obtain the duration of apparent colocalisation ($\tau_{\text{apparent colocalisation}}$), using the equation $[\tau_{\text{observed}}^{-1} - 2\tau_{\text{bleach}}^{-1}]^{-1}$ ^{4,11,29}. The effective dimer lifetime or the effective binding lifetime of proteins, τ , was evaluated by subtracting the duration of incidental colocalisation (without specific molecular interactions; $\tau_{\text{incidental}}$) from $\tau_{\text{apparent colocalisation}}$. For details, see our previous publications^{4,29}. $\tau_{\text{incidental}}$ was evaluated by using a monomer reference molecule, ACP linked to the transmembrane domain of the low-density lipoprotein receptor, called ACP-TM²⁹, and was found to be 19 ± 0.46 ms (exponential lifetime; $n = 241$ examined dimers; 39 examined movies; No. of Freedoms = 97).

357

358 **Observations of the recruitment of $G\alpha_s$ and $G\beta\gamma$ to β AR monomers and dimers**

359 The recruitment of $G\alpha_s$ and $G\beta\gamma$ to β AR monomers and dimers was detected by observing
360 the colocalisation-codiffusion of mGFP-conjugated $G\alpha_s$ and $G\beta\gamma$ with ATTO594-labelled
361 β 2AR monomers and dimers, at the level of single molecules. The colocalisation events
362 lasting for longer than 66 ms (2 video frames) were analysed. The frequency of incidental
363 colocalisation was estimated by superimposing an image sequence with one color on
364 another image sequence with a different color that was artificially shifted by ~ 500 nm
365 (horizontally), and was subtracted. The recruitment frequencies of $G\alpha_s$ and $G\beta\gamma$ to β AR
366 monomers and dimers were normalised by the number densities of $G\alpha_s$ (or $G\beta\gamma$) and β 2AR
367 protomers in the PM (per sec per μm^4).

368

The BiFC method for examining the formation of β 2AR dimers (the interaction of two β 2AR molecules at the molecular level)

The YFP-based BiFC(N) and BiFC(C) moieties employed previously^{4,37} were respectively fused to the C terminus of ACP- β 2AR (**Extended Data Fig. 1**) in the pEGFP-N1 vector (Invitrogen; the cDNA region encoding EGFP was removed). These proteins were co-expressed in L cells, by transfection with LipofectAMINE LTX (Life Technologies) according to the manufacturer's recommendations, and then ATTO594 was conjugated to the ACP moieties of ACP- β 2AR-BiFC(N) and ACP- β 2AR-BiFC(C). As the monomer reference molecule for the BiFC data, ACP-TM was employed and the YFP-based BiFC(N) and BiFC(C) moieties were fused to the C terminus of ACP-TM, as previously described⁴. Using dual-color single-molecule imaging (ATTO594 and mGFP), the number densities of the expressed molecules (the sum of the number densities of ACP-conjugated molecules) and those of the fluorescence spots of reconstituted BiFC molecules (spots/ μm^2) were determined.

Evaluation of the changes of the cytosolic cAMP levels

The amounts of cAMP in the cytosol were measured by using the time-resolved fluorescence resonance energy transfer (TR-FRET) kit from Perkin Elmer (EnVision Xcite), according to the manufacturer's recommendations. Since the L cells used for single-molecule experiments expressed very low levels of β 2AR, and thus the basal level of cAMP in the resting state was below the detection limit, we first established a stable L-cell line expressing β 2AR at high levels³⁸, and used these cells to evaluate the changes of the cAMP levels in the cytosol before and after the additions of the agonist and inverse agonists. The drug treatments were performed as described at the end of the subsection, "Cell culture, cDNA construction and expression in L cells, and treatment of cells with agonists and inverse agonists".

Generation of artificial β 2AR dimers and observations of Ca^{2+} mobilisation

To artificially generate stable homo-dimers of β 2AR, we employed a chemical dimerising

397 system based on the FK506-binding protein (FKBP)-ligand interface³⁹. First, the cDNA
 398 encoding Halo7-β2AR-FKBP_F36V (β2AR-FKBP) was cloned into the expression plasmid
 399 vector pEGFP-N1 (Clontech) after the removal of the cDNA region encoding EGFP, and then
 400 the cDNA encoding FKBP_F36V was replaced by the cDNA encoding FKBP (obtained from
 401 the YFP-FKBP cDNA, the Addgene plasmid 20175 donated by T. Meyer⁴⁰). Halo7-β2AR-FKBP
 402 expressed in L cells was fluorescently labelled by incubating the cells for 24 - 48 h with 5 nM
 403 Halo-tag conjugated with SaraFluor 650T (Goryo-Kayaku;
 404 SaraFluor650T-Halo7-β2AR-FKBP). The crosslinker for FKBP, AP20187 (Takara), was added
 405 at a final concentration of 5 nM during the image acquisition, and dimer formation was
 406 detected at the level of single molecules.

407 For Ca²⁺ mobilisation assays, a freshly-prepared 1 mg/ml DMSO solution of Fluo4-AM
 408 (Dojindo) was added to HBSS buffered with 10 mM
 409 (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.4 (H-HBSS), containing 1.25
 410 mM Probenecid (Dojindo) and 0.04 % (w/v) Pluronic F127 (Dojindo) (loading buffer), to
 411 obtain a final Fluo4-AM concentration of 5 µg/ml (4.6 µM), basically following the
 412 manufacturer's recommendations. The L cells prepared as above for detecting dimers of
 413 SaraFluor650T-Halo7-β2AR-FKBP at the single-molecule level were incubated with the
 414 loading buffer at 37°C for 1 h and washed with H-HBSS three times, and then 500 µl
 415 H-HBSS was added for epi-fluorescence microscopic observations. Fluo4 fluorescence was
 416 recorded at 2 Hz. The stable dimers of SaraFluor650T-Halo7-β2AR-FKBP were induced by
 417 the addition of 5 nM AP20187, as described above. To determine the saturation levels of the
 418 fluorescence signal intensity of Fluo4 at higher concentrations of Ca²⁺, 1 µM (final
 419 concentration) ionomycin (Wako) was added to the cells to equalise the intracellular Ca²⁺
 420 concentrations with that outside the cells (1.3 mM).

421

422 **Software and statistical analysis**

423 Superimpositions of image sequences obtained from dual-color single fluorescent molecule
 424 observations and single fluorescent spot tracking were performed using C++-based

computer programs produced in-house, as described previously^{30,32}. Statistical analysis was performed by the two-sided Welch's t test (except for the data shown in **Extended Data Fig. 5c**, where the one-sided t test was used) or the Brunner-Munzel statistical test, by using the R Studio Software (R Foundation for Statistical Computing, <https://www.R-project.org/>). *P* values less than 0.05 were considered to be statistically significant. Linear and non-linear curve fittings were performed by using OriginPro 9.1 for Windows, with appropriate scripts.

Data and code availability

All data generated or analysed for this study are available within the paper and its associated Extended Data Figures and Tables. All other data presented and software codes are available upon request from the corresponding author. A **Reporting Summary** for this paper is available.

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Fig. 1

Fig. 1. β2AR dynamically interconverts between monomers and transient homodimers.

Mean, SEM, the number of experiments, and other statistical parameters are summarised in

Extended Data Table 1.

(a) A typical snapshot sequence showing transient homo-dimerisation of β2AR molecules. Two molecules became colocalised at frame 2 (67 ms), diffused together for 133 ms (colocalisation), and then separated.

(b) Histogram showing the distribution of the durations of the WT-β2AR colocalisation events (homo-dimers). Note that the τ_{observed} is the value before corrections for photobleaching and incidental colocalisations.

(c) Summary of the dimer lifetimes (after corrections) of WT and mutant β2AR obtained before and after the additions of various reagents. ** and *n.s.*: statistically significant and non-significant with *P* values smaller or greater than 0.05, respectively. This convention is used throughout this report.

Fig. 2

Fig. 2. Determining the 2D-KD of β_2 AR dimers with/without an inverse agonist and the BiFC detection of β_2 AR dimers. Mean, SEM, the number of experiments, and other statistical parameters are summarised in **Extended Data Table 2**.

The number density of β_2 AR molecules in true dimers (after subtraction of incidental overlap of fluorescent spots; $\pm$ inverse agonist ICI-118,551; curves represent the fitting results using Eq. 5 in Ref. 4) **(a)** and the number densities of the BiFC spots for ACP- β_2 AR-YFP(N/C) and ACP-TM-YFP(N/C) (monomer reference molecule) **(b)**, plotted as a function of the number density of expressed molecules (ND_{mol}). In **b**, the lines are provided to facilitate visualisation.

Fig. 3

Fig. 3. Non-stimulated β 2AR homo-dimers recruit trimeric G-proteins: a process

suppressed by inverse agonists. Mean, SEM, the number of experiments, and other statistical parameters are summarised in **Extended Data Table 3**.

(a) A typical snapshot image sequence showing that a trimeric G-protein G α s subunit transiently binds to a β 2AR monomer.

(b, c) The inverse agonists, ICI-118,551 and timolol, inhibitors of β 2AR basal constitutive activities, blocked the recruitment of trimeric G-proteins to β 2AR homo-dimers, but not that to monomers. G α s **(b)** and G β γ **(c)** colocalisation frequencies with β 2AR monomers and homo-dimers, normalised by the number densities of G α s (or G β γ) and β 2AR protomers (per sec per μm^4).