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, β2-adrenergic receptor (β2AR), elicit low levels of basal constitutive signals without agonist stimulation, which function in normal development and various diseases[1-3]. 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 quantification[4] determined the dissociation constant of β2AR 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 β2AR 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.

β2AR forms metastable homo-dimers

Single-molecule imaging-tracking revealed that β2AR (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 β2AR 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 $\tau_{observed}$, 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 β2AR. Together, these results demonstrate that the β2AR 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 β2AR dimers

Using single-molecule-based "super-quantification" method previously developed by us[4], we obtained the 2-dimensional dissociation constant (2D-K_D) of β2AR 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, β2AR is expressed at number densities of 16 ~ 260 copies/μm^2, depending on the cell type[8,9]. From the 2D-K_D determined here, we conclude that 12 ~ 240 copies/μm^2 exist as dimers and 4 ~ 20 copies/μm^2 exist as monomers at any moment; *i.e.*, 75 ~ 92% of β2AR 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 copies exist in the PM of a single neutrophil[10]. Assuming that the neutrophil surface area is 1,300 μm^2 (approximated by a sphere with a radius of 10 μm, 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

79 as dimers; namely, 54% of FPR molecules are dimers, which is about two-thirds of the dimer fraction
80 of β2AR. Note that, even under the conditions where the dimer population dominates (*i.e.*, at higher
81 expression levels), the dimer lifetimes do not change and remain on the order of 0.1 s. Only the
82 monomer lifetimes are shortened at higher expression levels.
83
84 **Direct detection of β2AR dimers by the BiFC method**
85 Thus far, we have detected dimers by the colocalisation of two single molecules, which reveals the
86 close encounters of molecules in the space scale of 220 nm, a distance much greater than the
87 molecular scale. Therefore, in this method, the incidental approaches of two molecules were
88 evaluated and subtracted. To ascertain the molecular-level interactions (dimers), we employed
89 YFP-based bimolecular fluorescence complementation (BiFC; meanwhile, our attempt to detect
90 FRET did not work, perhaps because the distance between the probes attached to two C-termini was
91 too far for FRET)[4,11]. In BiFC, two potentially interacting proteins are fused to the N- and C-terminal
92 half-molecules of YFP. If the proteins of interest form dimers, then YFP may be reconstituted and
93 emit fluorescence signals (see **Methods**). Thus, if BiFC occurs, it would lend strong support for the
94 formation of β2AR dimers.
95 To quantitatively examine BiFC, we varied the expression levels of β2AR. With an increase in
96 the number density of expressed molecules (determined by the signal from ATTO594 bound to ACP;
97 the ATTO594/ACP ratio is >0.95[12]), the number density of the BiFC spots of β2AR increased,
98 whereas the BiFC-spot number density of the monomer reference molecule, ACP-TM, increased only
99 slightly (**Fig. 2b; Extended Data Fig. 2c**). This result indicates that β2AR forms directly-bound
100 dimers.
101
102 **Inverse agonists reduce the β2AR dimer affinity**
103 Treatments with the inverse agonists ICI-118,551 (ICI) and timolol reduced the β2AR dimer lifetime
104 (0.74x and 0.69x, respectively; **Fig. 1c, Extended Data Fig. 2d**), whereas ICI increased the 2D-K_D
105 of β2AR by a factor of 2.1 (lowered the β2AR dimer affinity; **Fig. 1c**), indicating that inverse agonists
106 reduce the β2AR dimer affinity. Meanwhile, the addition of the agonist isoproterenol (Iso) prolonged

107 the dimer lifetime significantly (1.55x) (**Fig. 1c; Extended Data Fig. 2d**). Cholesterol reportedly
108 modulates the functions of some GPCRs[13], but here we found that mild cholesterol depletion by
109 methyl-β-cyclodextrin (MβCD) treatment failed to affect the β2AR dimer lifetime (**Fig. 1c**).
110 Since the inverse agonists suppressed the basal constitutive GPCR signaling, and since we
111 found that they also reduced the β2AR dimer lifetime, we next examined the dimer lifetimes of the
112 β2AR mutants with high and low basal activities in the resting state (HBA and LBA mutants,
113 representing E268A and C327R mutants, respectively[14,15]). The HBA mutant exhibited a 1.78x longer
114 dimer lifetime, and interestingly, after the inverse-agonist addition, it was reduced to the dimer
115 lifetime of the wild-type receptor (**Fig. 1c; Extended Data Fig. 2d**). Meanwhile, the LBA mutant
116 exhibited a 0.74x shorter dimer lifetime than that of the wild-type receptor, and the agonist addition
117 did not affect it (**Fig. 1c; Extended Data Fig. 2d**).
118 Strikingly, the dimer lifetimes of WT-β2AR after the addition of inverse agonists were quite
119 comparable to those of the LBA mutant, and the dimer lifetime of WT-β2AR after the addition of the
120 agonist isoproterenol was comparable to that of the HBA mutant. These results suggest that the
121 β2AR conformations would be quite different after the binding of the inverse agonists and the
122 agonist isoproterenol, as compared with those before their additions[16], possibly resembling the
123 conformations of the LBA and HBA mutants, respectively, and detectable as changes in the
124 homo-dimer lifetimes.
125
126 **The basal constitutive activity of β2AR**
127 We confirmed the basal constitutive activity of β2AR, as well as the effects of the β2AR agonist
128 isoproterenol and the inverse agonists ICI-118,551 and timolol, by measuring the changes in the
129 cytoplasmic cAMP levels in the L-cell line stably expressing β2AR at higher levels (**Extended Data**
130 **Fig. 4, Extended Data Table 4**). The addition of the agonist isoproterenol to the cells increased
131 the cAMP level by a factor of ~4, relative to the control. Meanwhile, the addition of the inverse
132 agonists ICI-118,551 and timolol reduced the cAMP levels by factors of 1.9 and 1.3 (53 and 76% of
133 the control), respectively, showing the presence of the basal constitutive activity of β2AR and the
134 effectiveness of the inverse agonists, ICI-118,551 and timolol, for strongly reducing this basal

135 activity.

136

137 **Trimeric G proteins are recruited to both β2AR monomers and dimers**

138 The presence of the basal constitutive activity of β2AR suggests that a stimulatory trimeric G-protein
139 might be recruited to β2AR in the resting state[17]. Therefore, we examined whether Gαs and Gβ
140 (conjugated with mGFP) are recruited to β2AR monomers or dimers, by examining their
141 colocalisations. Using L cells expressing both β2AR (ATTO594-ACP-β2AR) and Gαs or Gβ, we found
142 that, even in resting cells, Gαs and Gβ were recruited to both β2AR monomers and dimers (**Fig. 3a;**
143 **Supplementary Videos 1 and 2**).

144 The recruitment frequencies of Gαs and Gβ to β2AR monomers and dimers were measured
145 and then normalised by the number densities of Gαs (or Gβγ) and β2AR protomers in the PM
146 (normalised frequencies, with a dimension of per sec per μm^4; **Fig. 3b, c; Extended Data Table**
147 **3**)[18]. In resting cells, both Gαs and Gβ molecules were recruited to β2AR monomers or dimers
148 equally well, suggesting that (non-stimulated) β2AR monomers and/or dimers might induce
149 intracellular Gs-dependent signals even in the resting state, which would lead to the basal
150 constitutive activity of β2AR.

151 The exponential dwell lifetimes of G proteins on β2AR were on the order of 50 ~ 60 ms for all
152 of the combinations of Gαs and Gβ molecules vs β2AR monomers and dimers (**Extended Data Fig.**
153 **5**). However, these durations are only the maximal estimates of the dwell lifetimes of Gαs and Gβ
154 molecules on β2AR monomers and dimers, due to the present instrumental limitations of detecting
155 short binding durations.

156 Notably, the treatment of the cells with the agonist, isoproterenol, did not change the
157 recruitment frequencies in any combinations of Gαs and Gβ vs β2AR monomers and dimers,
158 although it significantly enhanced the cytoplasmic cAMP level (**Extended Data Fig. 4, Extended**
159 **Data Table 4**). This result is quite surprising, but it suggests that the isoproterenol-induced β2AR
160 conformation enhanced its GEF activity, rather than increasing the on-rate of G protein binding to
161 β2AR.

162

163 **Inverse agonists specifically block G-protein recruitment to β2AR dimers**

164 Next, we examined the effects of the inverse agonists. The recruitment of Gαs and Gβ to β2AR
165 dimers was almost completely blocked, whereas that to β2AR monomers was unaffected (**Fig. 3b,**
166 **c**). This result unequivocally shows that β2AR dimers are responsible for the basal constitutive
167 activity of β2AR. Furthermore, it demonstrates that the inverse agonists work by blocking the
168 recruitment of stimulatory G-proteins to β2AR dimers, perhaps by inducing β2AR conformational
169 changes in the binding sites for G-proteins, rather than blocking the β2AR dimerisation (although, as
170 already described, the dimer affinity of the inverse agonist-bound β2AR is lower, resembling the LBA
171 mutant, and thus inverse agonists might change the GPCR conformations in two ways: lowering the
172 GPCR dimer affinity and modulating the G-protein binding sites).

173 To further confirm that β2AR dimers trigger downstream signaling without an agonist, and to
174 detect the basal dimer signal at very low β2AR expression levels used for single-molecule
175 experiments (generally <1 copy/μm^2, which is approximately comparable to <3,000 copies in the
176 PM/cell), we examined whether artificially-induced stable β2AR dimers could elicit the downstream
177 signal. To detect the downstream signal at very low expression levels of β2AR, which are necessary
178 for detecting dimers by single-molecule imaging, and also to detect the signal in a different manner,
179 rather than observing the cAMP level again (**Extended Data Fig. 4**), we examined the intracellular
180 Ca^{2+} mobilisation, which could occur further downstream from the cAMP amplification[19]. The
181 induction of the artificially-induced stable β2AR (conjugated to FKBP) dimers by the FKBP-A20187
182 dimerisation system indeed elicited Ca^{2+} mobilisation (**Extended Data Fig. 6**). This result further
183 confirmed that β2AR dimers could trigger the downstream signals without agonist stimulation.

184

185 **Discussion**

186 All of the class-A GPCRs examined thus far, including β2AR examined here and by others[5], FPR, D2
187 dopamine receptor, M1 muscarinic receptor, and α1AR, form metastable dimers with lifetimes on the
188 order of 0.1 s in the non-stimulated state[4-7]. Therefore, we propose that the dynamic equilibrium
189 between monomers and homo-dimers is a common characteristic property of the class-A GPCRs.
190 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 diseases[1-3]. 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 β2AR 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 Gαs and Gβ to β2AR dimers, without affecting their
203 recruitment frequencies to β2AR monomers (**Fig. 3b, c**). The third result unequivocally shows that
204 the G proteins bound to β2AR dimers, and not monomers, in the resting state are responsible for
205 triggering the β2AR's basal constitutive activity, which is sensitive to inverse agonism. It further
206 suggests that the binding of the inverse agonists to β2AR induces conformational changes that
207 decrease the β2AR 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**
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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 β2AR[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 β2AR 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 Gαs, the mGFP cDNA was inserted between the region encoding the
271 71st and 82nd amino acids of mouse Gαs (Origene) in the pcDNA3.1 (+) vector
272 (Invitrogen)[26], and for that to Gα, mGFP was fused to the N terminus of bovine Gα1 (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 mmϕ with a glass window of 12 mmϕ,
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

286 (P-HBSS), at concentrations of 20, 200, and 200 μM, respectively. For the additions to the
287 cells, 1 ml solutions of these reagents were added to the cells in 1 ml of the Ham's F12
288 medium described above, providing the final concentrations of 10 μM isoproterenol, 100 μM
289 ICI-118,551, and 100 μM timolol.
290

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

292 ATTO594-Coenzyme A was custom-synthesised by Shinsei Kagaku, by conjugating
293 ATTO594-maleimide (ATTO-TECH) to Coenzyme A-SH (New England Biolabs), followed by
294 HPLC purification. The chemical structure of ATTO594-Coenzyme A was confirmed by liquid
295 chromatography-mass spectrometry (LC-MS; LCMS-2010A EV, Shimadzu). The ACP-tagged
296 β2AR expressed in the PM of the L cells was labelled with ATTO594, by incubating the cells
297 with 2 μM ATTO594-Coenzyme A and 1 μM phosphopantetheine transferase (New England
298 Biolabs) in Ham's F12 medium without FBS at ~25°C for 20 min. These conditions have
299 been known to achieve ~95% labelling of ACP[25]. The cells were washed with P-HBSS three
300 times, and then subjected to microscope observations. The cells exhibiting the presence of
301 typically ~1 ATTO594-ACP-B2AR spot/μm^2 in the PM were used for single-molecule imaging
302 experiments.
303

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

305 The fluorescently-labelled ACP-β2AR expressed in the bottom PM was observed at the level
306 of single molecules at 37°C, using home-built objective-lens-type total internal reflection
307 fluorescence microscopes based on Olympus IX-70, Olympus IX-81, or Nikon TE-2000
308 inverted microscopes[4,28-30], with a 100x objective lens, as optimised for the present research.
309 Fluorescence images were projected onto a two-stage microchannel plate intensifier
310 (C8600-03, Hamamatsu Photonics), coupled to an electron bombardment CCD camera
311 (C7190-23; Hamamatsu Photonics) or an sCMOS camera (ORCA-Flash 4.0; Hamamatsu
312 Photonics) operated at 30 Hz. For observations at a frame rate of 250 Hz, a CMOS camera
313 (FASTCAM 1024PCI-II, Photron) was employed[31]. Fluorescent spots were identified by using

314 an in-house computer program, as described previously[4]. The positions (x and y
315 coordinates) of all of the observed single fluorescent-molecules were determined by a
316 computer program that employed the method developed previously[32,33]. For the
317 simultaneous dual-color single-molecule imaging, the images were spectrally split into two
318 emission arms, each containing the same image intensifier and camera.
319 To maintain the cells at 37˚C during the microscope observations, the entire
320 microscope, except for the two detection arms and the excitation arm, was placed in a
321 home-built microscope environment chamber made with thermo-insulating transparent
322 vinyl sheets, and three heating circulators (SKH0-112-OT, Kokensya) were appropriately
323 placed in the chamber to supply warmed air with slow circulation[30,34,35]. For further
324 stabilisation, a plate heater (INUG2, Tokai Hit) was mounted on the microscope stage. The
325 temperature of the surrounding air just above the stage was continually monitored during
326 the observations with a digital thermometer (DT-312, Intermedical).
327
328 **Detection of colocalisation-codiffusion, and evaluations of β2AR dimer lifetimes**
329 **and two-dimensional dissociation constants of β2AR dimers**
330 The number densities of β2AR molecules existing as (true) dimers and the number density
331 of the expressed β2AR molecules were determined by single-molecule imaging of
332 ATTO594-ACP-β2AR[4]. Colocalisations of two fluorescent spots (molecules) of the same color
333 (the same ATTO594 probe) or those of two different colors (YFP for BiFC and ATTO594)
334 were detected when the two fluorescent molecules became localised within 220 nm of each
335 other[4,30]. The number densities of incidental colocalisations at given molecular densities
336 were subtracted[4]. The two-dimensional dissociation constant (2D-K_D) of β2AR in the PM was
337 determined by using the method previously developed by us[4].
338 To directly detect the actual binding of two β2AR molecules, forming homo-dimers, we
339 employed the bimolecular fluorescence complementation (BiFC) method[4,36]. The details of
340 the BiFC method are described in the next subsection.

To determine the lifetime of β2AR dimers using single color observations (with ATTO594-ACP-β2AR), 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 (τ_{bleach} = 7.21 ± 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 ($\tau_{apparent\ colocalisation}$), using the equation $[\tau_{observed}^{-1} - 2\tau_{bleach}^{-1}]^{-1}$ [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; $\tau_{incidental}$) from $\tau_{apparent\ colocalisation}$. For details, see our previous publications[4,29]. $\tau_{incidental}$ was evaluated by using a monomer reference molecule, ACP linked to the transmembrane domain of the low-density lipoprotein receptor, called ACP-TM[29], and was found to be 19 ± 0.46 ms (exponential lifetime; n = 241 examined dimers; 39 examined movies; No. of Freedoms = 97).

Observations of the recruitment of Gαs and Gβγ to βAR monomers and dimers

The recruitment of Gαs and Gβγ to βAR monomers and dimers was detected by observing the colocalisation-codiffusion of mGFP-conjugated Gαs and Gβγ with ATTO594-labelled β2AR monomers and dimers, at the level of single molecules. The colocalisation events lasting for longer than 66 ms (2 video frames) were analysed. The frequency of incidental colocalisation was estimated by superimposing an image sequence with one color on another image sequence with a different color that was artificially shifted by ~500 nm (horizontally), and was subtracted. The recruitment frequencies of Gαs and Gβγ to βAR monomers and dimers were normalised by the number densities of Gαs (or Gβγ) and β2AR protomers in the PM (per sec per μm^4).

369 **The BiFC method for examining the formation of β2AR dimers (the interaction of**
370 **two β2AR molecules at the molecular level)**
371 The YFP-based BiFC(N) and BiFC(C) moieties employed previously[4,37] were respectively
372 fused to the C terminus of ACP-β2AR (**Extended Data Fig. 1**) in the pEGFP-N1 vector
373 (Invitrogen; the cDNA region encoding EGFP was removed). These proteins were
374 co-expressed in L cells, by transfection with LipofectAMINE LTX (Life Technologies)
375 according to the manufacturer's recommendations, and then ATTO594 was conjugated to
376 the ACP moieties of ACP-β2AR-BiFC(N) and ACP-β2AR-BiFC(C). As the monomer reference
377 molecule for the BiFC data, ACP-TM was employed and the YFP-based BiFC(N) and BiFC(C)
378 moieties were fused to the C terminus of ACP-TM, as previously described[4]. Using dual-color
379 single-molecule imaging (ATTO594 and mGFP), the number densities of the expressed
380 molecules (the sum of the number densities of ACP-conjugated molecules) and those of the
381 fluorescence spots of reconstituted BiFC molecules (spots/μm^2) were determined.
382
383 **Evaluation of the changes of the cytosolic cAMP levels**
384 The amounts of cAMP in the cytosol were measured by using the time-resolved fluorescence
385 resonance energy transfer (TR-FRET) kit from Perkin Elmer (EnVision Xcite), according to
386 the manufacturer's recommendations. Since the L cells used for single-molecule
387 experiments expressed very low levels of β2AR, and thus the basal level of cAMP in the
388 resting state was below the detection limit, we first established a stable L-cell line
389 expressing β2AR at high levels[38], and used these cells to evaluate the changes of the cAMP
390 levels in the cytosol before and after the additions of the agonist and inverse agonists. The
391 drug treatments were performed as described at the end of the subsection, "Cell culture,
392 cDNA construction and expression in L cells, and treatment of cells with agonists and inverse
393 agonists".
394
395 **Generation of artificial β2AR dimers and observations of Ca^{2+} mobilisation**
396 To artificially generate stable homo-dimers of β2AR, we employed a chemical dimerising

397 system based on the FK506-binding protein (FKBP)-ligand interface[39]. 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. Meyer[40]). 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 Ca^{2+} 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 Ca^{2+}, 1 μM (final
419 concentration) ionomycin (Wako) was added to the cells to equalise the intracellular Ca^{2+}
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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546
547

Fig. 1

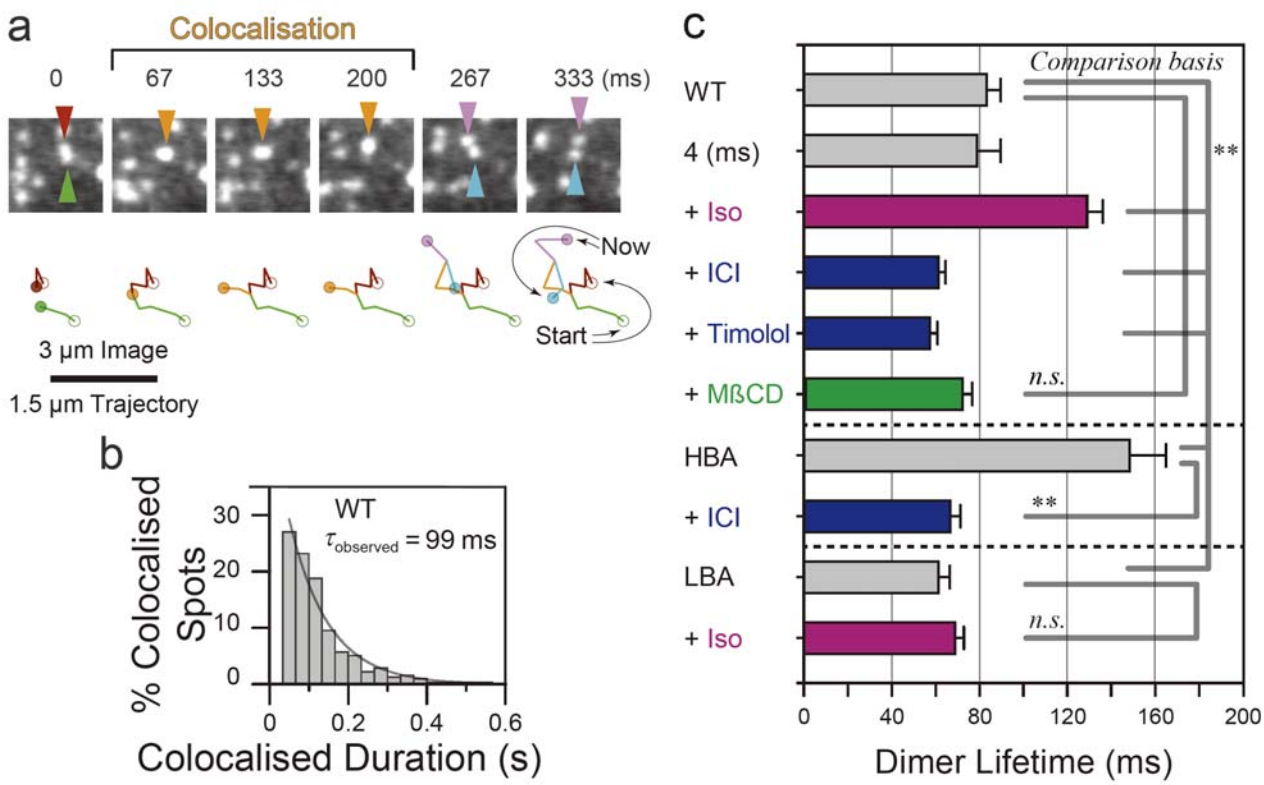

Fig. 1. β2AR dynamically interconverts between monomers and transient homodimers. Mean, SEM, the number of experiments, and other statistical parameters are summarised in **Extended Data Table 1**.

(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 $\tau_{observed}$ 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

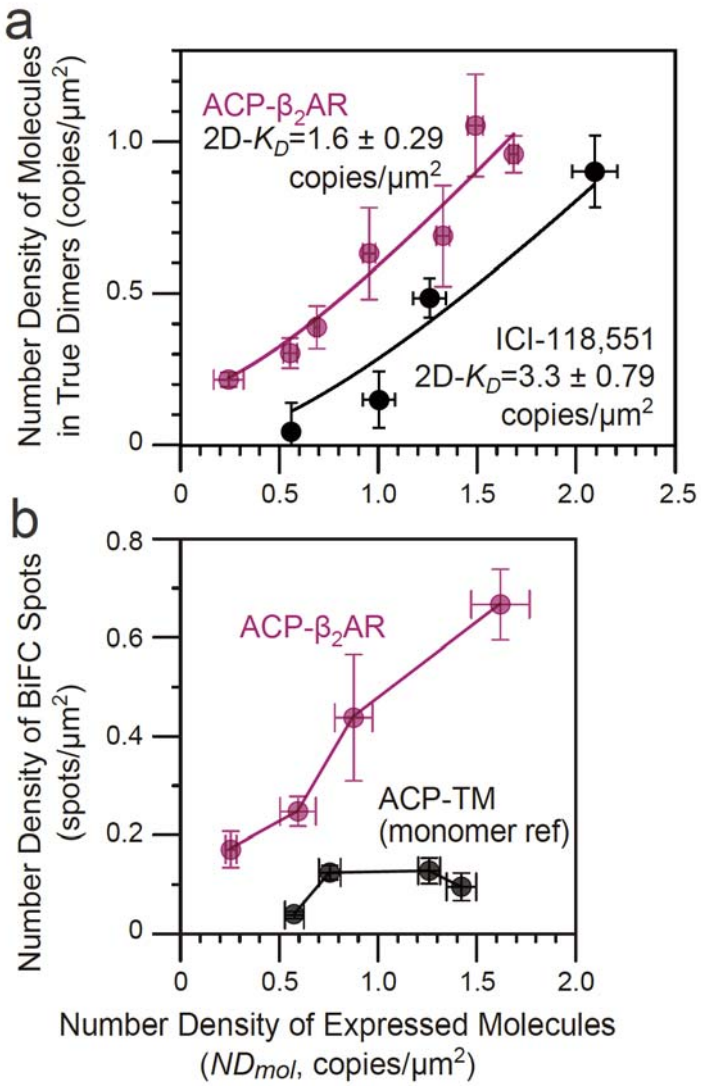

Fig. 2. Determining the 2D-*K*D of β2AR dimers with/without an inverse agonist and the BiFC detection of β2AR dimers. Mean, SEM, the number of experiments, and other statistical parameters are summarised in **Extended Data Table 2**.

The number density of β2AR molecules in true dimers (after subtraction of incidental overlap of fluorescent spots; ± 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-β2AR-YFP(N/C) and ACP-TM-YFP(N/C) (monomer reference molecule) **(b)**, plotted as a function of the number density of expressed molecules (*NDmol*). In **b**, the lines are provided to facilitate visualisation.

Fig. 3

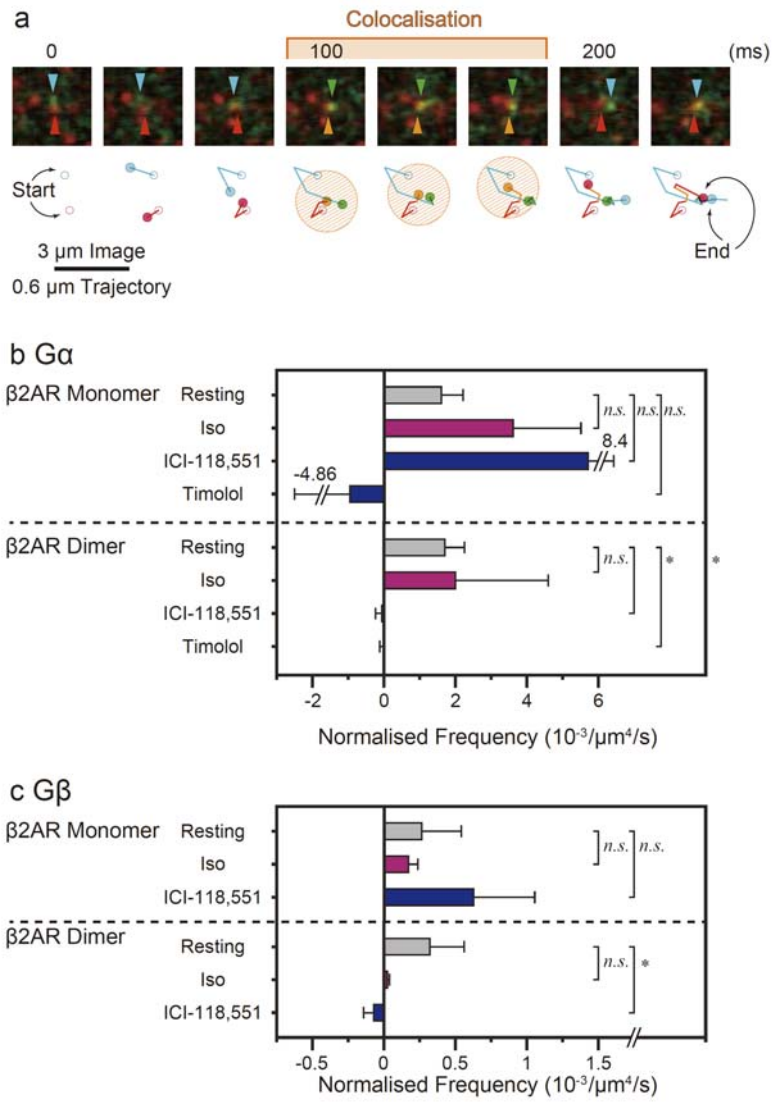

Fig. 3. Non-stimulated β2AR homo-dimers recruit trimeric G-proteins: a process suppressed by inverse agonists. Mean, SEM, the number of experiments, and other statistical parameters are summarised in **Extended Data Table 3**.

(a) A typical snapshot image sequence showing that a trimeric G-protein Gαs subunit transiently binds to a β2AR monomer.

(b, c) The inverse agonists, ICI-118,551 and timolol, inhibitors of β2AR basal constitutive activities, blocked the recruitment of trimeric G-proteins to β2AR homo-dimers, but not that to monomers. Gαs **(b)** and Gβγ **(c)** colocalisation frequencies with β2AR monomers and homo-dimers, normalised by the number densities of Gαs (or Gβγ) and β2AR protomers (per sec per μm^4).