Supporting Information

Single-molecule imaging reveals the stoichiometry change of β_2 -

adrenergic receptors by pharmacological biased ligand

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Materials and Methods

Cell culture and transfection.

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (50 mg/mL streptomycin, 50 U/mL penicillin) at 37 °C in 5% CO₂. Transfection was performed using Neofect (Neofect Biotechnologies, Beijing, China) according to manufacturer's instructions. Generally, cells growing in a 35-mm glass-bottom dish were transfected with 0.5 μ g β ₂AR-GFP plasmids or 0.5 μ g β ₂AR-mCherry, 0.5 μ g β ₂AR-flag and 0.35 μ g β -arrestin2-GFP plasmids in the serum-free and phenol red-free DMEM for 8 h. After 15 min stimulation, cells were fixed with 4% paraformaldehyde/PBS solution for 30 min or exposed to TIRFM objective directly for living cells imaging. For cells expressing β ₂AR-flag, they were sequentially incubated sequentially with anti-flag mouse antibody (Santa Cruz) overnight and the Alexa Fluor 647-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C before imaging. All drugs tested were purchased from Sigma.

Single Molecule Fluorescence Imaging.

The single-molecule imaging was using a TIRFM with an inverted Olympus IX71 microscope equipped with a total internal reflective fluorescence illuminator, a $100 \times /1.45$ NA Plan Apochromatic objective and an electron-multiplying charge-coupled device (EMCCD) camera (Andor iXon DU-897D BV). GFP, mCherry and flag tagged samples were excited at 488, 561 and 647 nm line of laser (Melles Griot, Carlsbad, CA, USA) respectively. The collected fluorescent signals were passed through the filter HQ 525/50, 617/73 and 700/75 (Chroma Technology) corresponding to 488, 561 and 647 nm excitation, and then directed to the EMCCD camera. The gain of the EMCCD camera was set at 300. Movies of 300 frames were acquired for each sample at a frame rate of 10 Hz.

Image analysis.

For analysis of single-molecule fluorescence intensity in a movie acquired from living cells, the background fluorescence was first subtracted from each frame using the rolling ball method in Image J software. Then the first frame of each movie was used for fluorescent spot (regions of interest) selection. The image was thresholded (five times of

the mean intensity of an area with no fluorescent spots), then filtered again with a userdefined program in Matlab.¹

To analyse the photobleaching steps of β_2 AR-GFP, regions of interest for bleaching analysis were selected according to previously reported.¹ The background fluorescence was firstly subtracted from the movie acquired from the fixed cells using the rolling ball method. Then the first five frames of the movie were averaged. The averaged image was thresholded and filtered with the method we developed. Finally, time courses of the integrated fluorescence intensity of regions of interest were extracted for bleaching analysis using the algorithm we developed.² Traces with erratic behavior and ambiguities (less than 20% of traces) were discarded.

The colocalization of β -arrestin2-GFP, the β_2AR -mCherry and β_2AR -flag was determined by using the Blobprob ImageJ plugin as previously reported.³

β-arrestin-dependent ERK 1/2 signalling and Gs-dependent cAMP analysis.

The cAMP accumulation and ERK 1/2 phosphorylation were measured with commercial human cAMP and pERK Elisa kits (Mlbio Biological Technology, Shanghai, China) according to the manufacturer's protocol. As shown in the Fig. S1, the full agonist isoproterenol activated both the two pathways, leading to increased cAMP production and ERK 1/2 phosphorylation. On the contrary, no cAMP accumulation and no pERK 1/2 were observed upon stimulation of the antagonist propranolol, indicating both G protein and β -arrestin dependent signalling were inactive. The β -arrestin biased ligand carvedilol, as expected, selectively promoted significant pERK 1/2 generation without cAMP production, suggesting it activated β -arrestin signalling from the receptor without eliciting G protein activation.

Silencing of β-arrestin1 and -2 expression.

The siRNA sequence targeting β -arrestin1 and -2 were 5'-AAAGCCUUCUGCGCGGAGAAU-3' and 5'-AAGGACCGCAAAGUGUUUGUG-3' corresponding to positions 439-459 and 148-168 relative to the start codon, respectively.⁴ The non-silencing RNA duplex 5'-AAUUCUCCGAACGUGUCACGU-3' was used as control. The RNA oligos were synthesized by GenePharma Co. (Suzhou, China). Target gene knockdown was achieved by transfection of 100 pmol siRNA and 5 μ L Lipofectamine2000 (Invitrogen) in 200 ml DMEM without serum in early passage HEK293 cells that plated in 6-well dishes. The cells were split and seeded into 35-mm glass-bottom dishes 48 hours later for further assays.

The gene knockdown was validated by Western blot and immunofluorescence analysis. For Western Blot analysis, cell lysates underwent protein determination by use of a bicinchoninic acid (BCA) protein assay kit (Biomiga) first. Then 50 mg proteins were loaded onto 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane (Pall) for immunoblotting. The nitrocellulose membrane were incubated with diluted β -arrestin1 and -2 primary antibodies (Proteintech, 1:500) overnight at 4° C. After washing, the membranes was immersed in 5% skim milk with HRP labelled secondary antibodies for 2 hours at room temperature. The HRP signal was analysed using the GeneGnome Bio Imaging System (Syngene). The expression of GAPDH was used as an internal control.

For immunofluorescence assays, HEK293 cells stably expressing endogenous β arrestin and silenced β -arrestin1 or -2 for 48 h were plated on 35-mm glass-bottom dishes. Then cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 min, and fixed with 4% formaldehyde in PBS for 30 min at room temperature, then incubated with β arrestin1 or -2 rabbit polyclonal antibody (Proteintech, 1:1000) and Alexa Fluor 488conjugated anti-rabbit secondary antibody (Invitrogen). Fluorescence images were collected using single line excitation (488 nm) with a confocal microscope (FluoView FV1000-IX81, Olympus, Japan) equipped with a 100×/1.40NA objective.

Single-molecule imaging using cell extracts.

HEK 293 cells were cultured in a 10-cm culture dish and transfected with 10 μ g β_2 AR-GFP for 8h. The cells were harvested when the coverage rate >90%. Crude membrane fractions were extracted using a membrane preparation kit (Applygen, Beijing) and then were lysed to get membrane protein. The extracted membrane proteins from cell lysates were solubilized in 2 ml PBS buffer firstly. Then the lysates were diluted 1000 to 10000-fold with PBS before imaging.

In order to obtain single-molecule imaging by TIRFM, the extracted membrane proteins from cell lysates were immobilized on the coverglass as we previously reported.^{1a} In brief, glass surfaces were first coated with biotin-BSA and then streptavidin, biotinylated anti-GFP molecules. This provided a reliable immobilization protocol for β_2 AR-GFP.

Statistical analysis.

We used Wilcoxon-test by SAS software to analyze the statistical significance.⁵ At least three independent experiments were carried out under each experimental condition to obtain data from single-molecule imaging, with 10-14 cells imaged in each experiment. Data were obtained from each cell after the stimulation by different drugs and compared to that of the basal cells.



Supporting Figures

Fig. S1 Detection of cAMP (A) and pERK 1/2 (B) in HEK293 cells. HEK293 cells transfected β_2 AR-GFP were stimulated with 10 μ M isoproterenol (Iso), carvedilol (Car) and propranolol (Pro) and cell lysates were analyzed for cAMP accumulation and pERK generation. Full agonist isoproterenol induced both cAMP accumulation and ERK activation, while β -arrestin-biased ligand carvedilol only caused pERK 1/2 generation. pERK was normalized to the maximal response induced by isoproterenol. (C) cAMP and (D) pERK 1/2 level were assessed in HEK293 cells transfected non-targeting control siRNA and β -arrestin1 or -2 depleted cells before and after isoproterenol and carvedilol stimulation. ***, *P* < 0.001 vs. control group (ctrl).



Fig. S2 Typical TIRFM single-molecule images of cell extract that diluted to 10000-fold with PBS in resting cells (A) and carvedilol stimulated cells (B). (C) Frequency of one and two-step bleaching events for β_2 AR-GFP before and after drug stimulation in cell extract diluted to 10000-fold. Data were obtained from three independent experiments, with 15 images for each sample in each experiment, and compared using Wilcoxon test. ***, P < 0.001.



Fig. S3 Distribution of the fluorescence intensity of diffraction-limited β_2AR -GFP spots before (A) and after 10 μ M isoproterenol (B), carvedilol (C) and propranolol (D) stimulation in HeLa cells. The biased ligand carvedilol stimulation enhanced dimerization of monomeric β_2AR -GFP on cell membrane while the full agonist isoproterenol and antagonist propranolol cannot.



Fig. S4 (A) The depletion of β -arrestin1 or -2 were validated by Western blot and confocal microscopy imaging. (B) Frequency of one and two-step bleaching events for β_2 AR-GFP before and after ligands stimulation in presence of non-targeting control siRNA (Ctrl).



Fig. S5 β -arrestin2-GFP was recruited to cell membrane and colocalized with β_2AR -mCherry after isoproterenol and carvedilol stimulation. HEK293 cells co-expressing β_2AR -mcherry and β -arrestin2-GFP were imaged by dualcolour TIRFM before (A) and after stimulation of 10 μ M isoproterenol (B), carvedilol (C) and propranolol (D). Quantification of colocalization of β_2AR -mCherry with β -arrestin2-GFP suggested ~47.8% and ~60.5% of β_2AR mCherry colocalized with β -arrestin2-GFP in the isoproterenol and carvedilol stimulated cells (Blobprob plugin, ImageJ).

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