Supporting Information

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SI Materials and Methods

Constructs and Reagents. Receptor and cortactin constructs have been described previously (1). Site-directed mutagenesis was performed with QuikChange (Stratagene) according to the manufacturer's instructions. HEK293 cells were purchased from American Type Culture Collection and cultured in DMEM (Thermo Scientific) with 10% FBS (Gibco). Cells were transfected using Effectene (Qiagen), and stably transfected cells were generated with Zeocin (Invitrogen) or Geneticin (Gibco). Isoproterenol hydrochloride (iso) and alprenolol hydrochloride (alp; Sigma-Aldrich) were prepared as 10 mM stocks in water, and KT-5720 (KT) and H-89 dihydrocholride (Santa Cruz Biotechnology) were prepared as 10 mM stocks in DMSO. All were used at a final concentration of 10 μM. Latrunculin A (lat-A; Cayman Chemical) was prepared at a concentration of 100 μg/mL of stock in ethanol and used at a final concentration of 10 μM.

Imaging and Analysis. Confocal images were obtained with an Andor Revolution XD spinning disk system on a Nikon Eclipse Ti inverted microscope equipped with a temperature-, humidity-, and CO_2-controlled chamber and a 100× total internal reflection fluorescence (TIRF) objective (Nikon). Cells were imaged live at 37 °C in Opti-MEM (Invitrogen) with 10% FBS and 40 mM Hepes. Solid-state 488-nm, 561-nm, or 647-nm lasers served as light sources. Images were acquired with an iXon+ 897 EM-CCD camera. Cells were imaged 5–15 min after the addition of (iso) and 1–5 min after the addition of KT-5720 or lat-A. Stacks and time-lapse images were collected as TIFF images and analyzed with ImageJ. Tubules and actin spots per endosome were counted double-blinded with scrambled file names. Concentration and protrusions from the body of the endosome, resulting in loss of circularity, were counted as tubules in all conditions.

For estimating total surface levels, cells were imaged with a 20× 0.75 numerical aperture (NA) objective under wide-field illumination for maximum collection efficiency. Images were collected at 1-min intervals. Four time points before iso were collected before the addition of agonist, and images were obtained for 10 min after the addition of agonist. After 10 min, KT or an equivalent volume of medium was added to cells. Alternatively, for washout, the iso-containing medium was removed, cells were washed once with plain medium, and medium containing 10 μM alp was added. Measured intensities were background-corrected and normalized to the average of the first four frames.

Total internal reflection fluorescence microscopy (TIR-FM) was performed using the same microscope system. Cells expressing pH-sensitive GFP (SpH)- transferrin receptor (TfR), SpH-beta 2 (B2), or SpH-beta 2 S345-6A (S > A) were imaged after 5 min of iso at 10 frames per second (fps) using a 60× 1.45 NA TIRF objective. Images were collected as TIFF images, and exocytic events were counted manually, double-blinded as above. For fixed cell immunofluorescence, HEK293 cells stably transfected with beta-2 adrenergic receptor (B2AR) or B2AR with mutated Ser-345 and 346 to alanines (B2ARS>A) were treated with iso, KT, the competitive PKA inhibitor Rp-cAMPS (Santa Cruz Biotechnology), or vehicle (DMSO) for 10 min in 10% FBS high-glucose DMEM. Cells were fixed in 4% paraformaldehyde and labeled with monocolonal anti-Flag M1 (1:1,000; Sigma-Aldrich) conjugated to Alexa Fluor 647 and rabbit anti–phospho-S346 B2AR (1:200; Assay BioTech) primary antibodies and goat anti-rabbit Alexa Fluor 488 conjugate antibody (1:10,000) secondary antibody. Confocal images were collected with a 60× 1.49 NA TIRF objective. All fluorescence measurements and quantitations were performed on images acquired directly from the camera without adjustments. Simple statistical analyses were performed in Microsoft Excel, and curve fitting was done using GraphPad Prism.

In Vitro Phosphorylation. The C-terminal 94 codons for the B2AR with or without the S > A mutations were inserted into pGEX-4T-1 plasmid vector. Phosphorylation reactions used 0.04 μg/μL protein kinase A (PKA) catalytic subunit from bovine heart (Sigma-Aldrich), 0.08 μg/μL GST-B2AR protein, and 50 μM ATP in kinase buffer [5 mM 3-(N-morpholino)propanesulfonic acid, 2.5 mM β-glycerophosphate, 1 mM EGTA, 4 mM $MgCl_2$, and 0.05 mM DTT] at room temperature for 30 min. Samples were immunoblotted with anti-GST and rabbit anti–phospho-S346 B2AR (1:500).

Signaling Assays. B2AR- or B2ARS>A-stable cells were transiently transfected with pGloSensor 20F (Promega). At 24 h after transfection, cells were passed at 10,000 cells per well to a poly-D-lysine–coated 96-well plate (Costar; Corning) and allowed to grow for 48 h. Cells were incubated at 37 °C for 1.5 h in CO_2-independent media (Gibco) supplemented with 10% FBS and 2% GloSensor cAMP Reagent stock solution, with 5 μM 3-isobutyl-1-methylxanthine added in the final 15 min. Luminescence was read on a Tecan Infinite M100 plate spectrometer in a temperature-controlled chamber at 35 °C. Each condition was performed in triplicate. Iso was added to wells at 10 μM final concentration. Control wells received the same number of washes with the same volume of additional media, but without iso. Washouts were performed by removing incubation medium, washing once with fresh medium without iso, and then adding medium containing 10 μM alp. Four separate experiments, each done in triplicate, were quantitated and analyzed using Microsoft Excel or GraphPad Prism.

1. Puthenveedu MA, et al. (2010) Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. *Cell* 143(5):761–773.

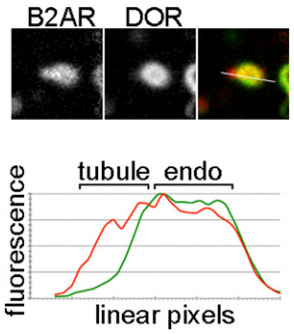


Fig. S1. B2AR tubules are not enriched for the delta opioid receptor (DOR), a nonrecycling GPCR. Shown is an example endosome from a cell coexpressing fluorescently tagged B2AR and DOR after exposure to iso and [D-Ala^2, D-Leu^5]-enkephalin (DADLE), the DOR agonist, revealing a B2AR tubule lacking DOR staining. The fluorescence line trace across the endosome and the tubule, along the line shown in the merge, confirms the lack of DOR in the tubule.

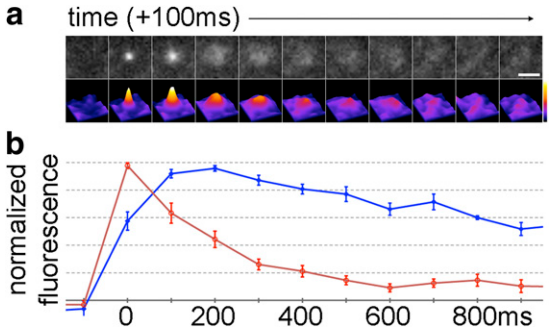


Fig. S2. Example of an individual B2AR insertion event. (*A*) Time series from a 10-fps TIR-FM movie showing an example SpH-B2AR insertion event, characterized by a sudden burst followed by diffusion of fluorescence. A heat map of the surface plot is shown at the bottom. (*B*) Quantitation of maximum (red) and average (blue) intensity fluorescence traces from multiple insertion events showing a transient increase in maximum fluorescence and sustained increase in average fluorescence, as expected for delivery of surface receptors.

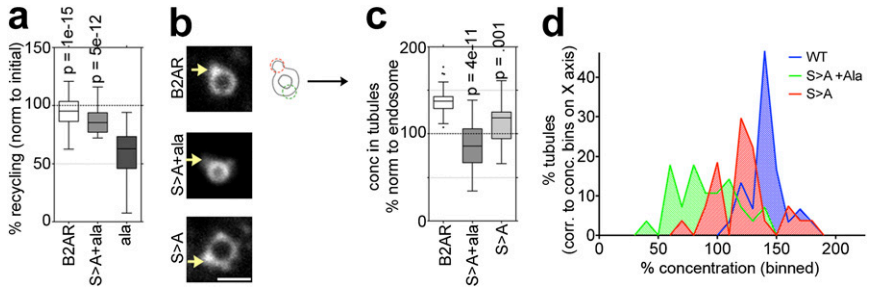


Fig. S3. B2AR S345-6A + C-terminal Ala (B2ARS>A+ala) is not concentrated in tubules, unlike B2AR and B2ARS>A. (*A*) Quantitation of SpH-B2AR recycling by confocal multifield analysis, as in Fig. 4 *A* and *B*. Total fluorescence of cells expressing SpH-tagged receptors was measured over 10 min of iso treatment followed by a 20-min washout with alp. Intensity after washout was normalized to the initial intensity. B2AR and B2ARS>A recycled significantly more than B2AR + C-terminal Ala (B2AR+ ala) ($n > 450$ cells for each construct). (*B*) Example endosomes in cells expressing the respective FLAG-tagged receptors with tubules indicated by arrows. A schematic of measurement to quantitate concentration of receptors in tubules vs. endosome is shown at the right. (*C*) Boxplots of receptor concentration in tubules, with 100% denoting the concentration on the endosome. *P* values from Mann–Whitney *U* tests compared with B2AR show significant decrease in concentration for B2ARS>A+ala. B2ARS>A shows a higher spread, presumably because of PSD95-Dlg1-zo-1 domain (PDZ) mediated concentration in sequence-dependent tubules and diffusion into bulk recycling tubules ($n = 30$ for B2AR, $n = 28$ for B2ARS>A+ala, $n = 27$ for B2ARS>A). (*D*) Receptor concentrations were binned into 10% bins, normalized to endosome concentration (100%). The percentage of total tubules falling into each bin is plotted. Most B2AR tubules showed a concentration above endosomes, averaging around 150%, whereas the B2ARS>A+ala tubules did not.

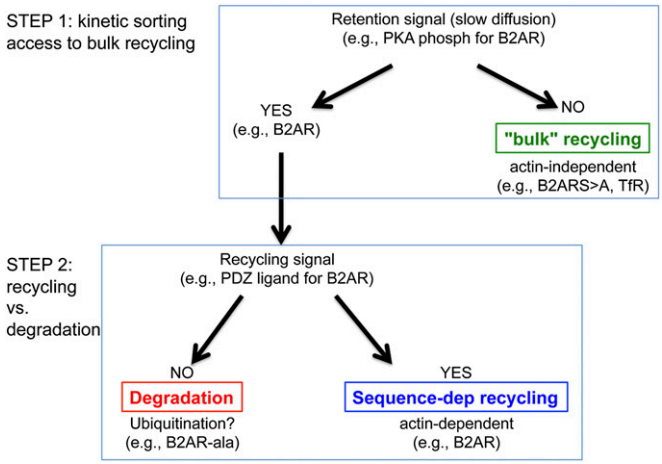


Fig. S4. Schematic of a hierarchical two-step model for B2AR sorting. In step 1, kinetic sorting, PKA-phosphorylated B2AR is kept from accessing bulk recycling by restricted diffusion on the endosomal membrane. Dephosphorylation releases this restriction and diverts receptors to the bulk (actin-independent) recycling tubules, suggesting that kinetic sorting can be physiologically regulated. In step 2, affinity-based concentration, B2AR is concentrated in sequence-dependent recycling tubules by PDZ interactions. Removal of PDZ ligand diverts B2AR to the lysosomal pathway, but it still cannot access the bulk recycling pathway, because it is phosphorylated. Removal of phosphorylation in addition to the PDZ ligand allows B2AR to access the bulk recycling pathway.

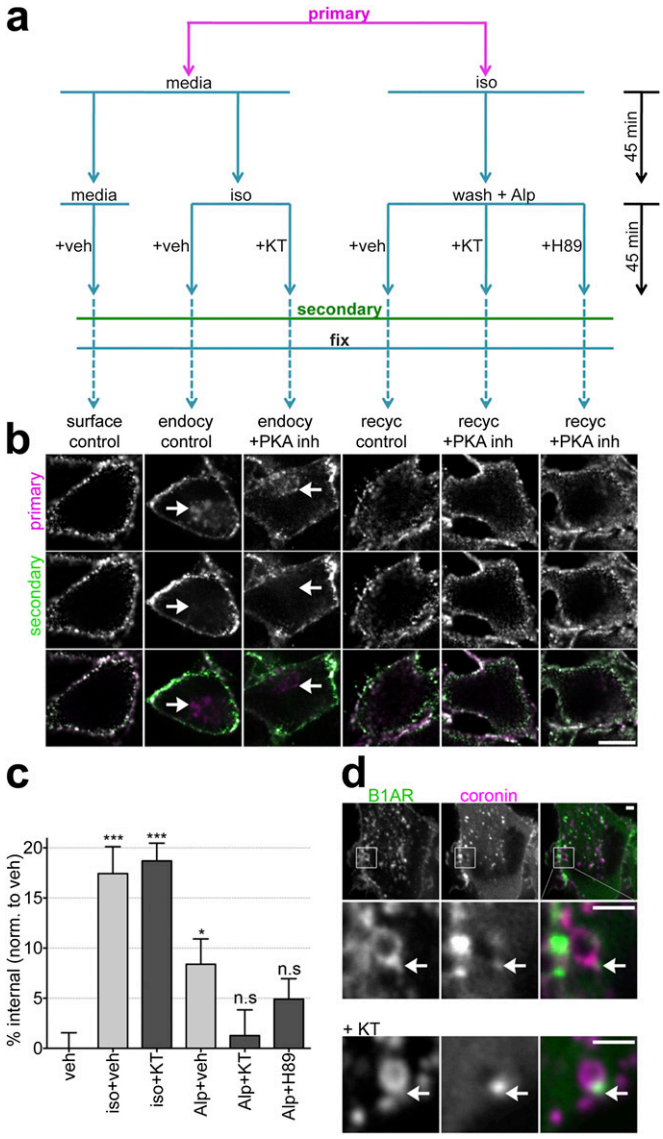
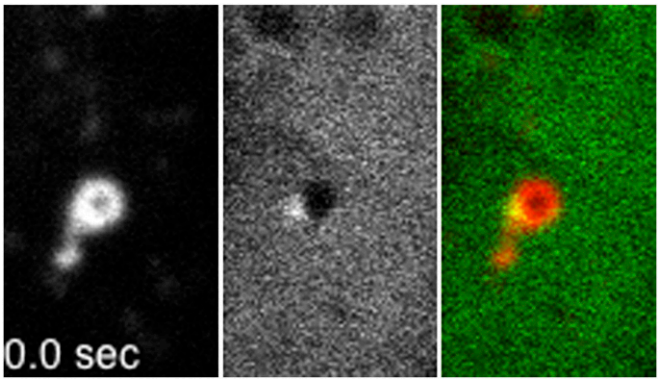
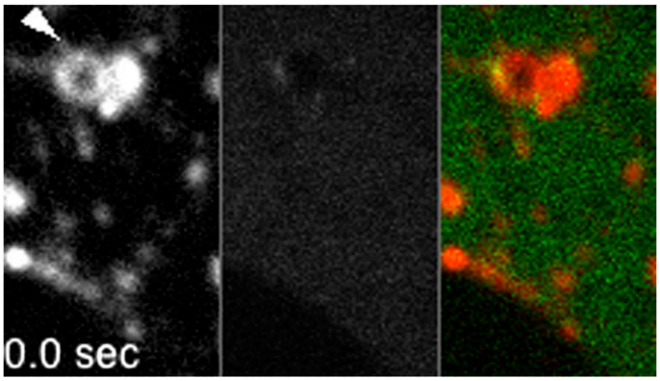


Fig. S5. PKA inhibition does not inhibit beta-1 adrenergic receptor (B1AR) recycling. (*A*) Schematic of a quantitative ratiometric assay to measure the effect of PKA inhibition on B1AR recycling in fixed cells. (*B*) Representative images of cells under each treatment, showing internalized receptors (white arrows) with iso, which are lost after washout. (Scale bar: 5 μm.) (*C*) Quantification of internalized and recycled receptors measured as a ratio of secondary antibody (final surface pool) to primary antibody (initial surface pool), normalized to vehicle-treated cells (veh). ***$P < 0.001$; *$P < 0.05$, Mann–Whitney *U* test compared with vehicle control. PKA inhibition by either KT or H89 increased surface recycling compared with alp alone. Error bars are SEM. $n = 30$ cells each condition. (*D*) Confocal images of live HEK293 cells (as in Fig. 1) coexpressing B1AR and coronin1C-GFP. The arrow indicates B1AR tubules decorated with coronin, which were present even after the addition of KT. (Scale bar: 1 μm.)



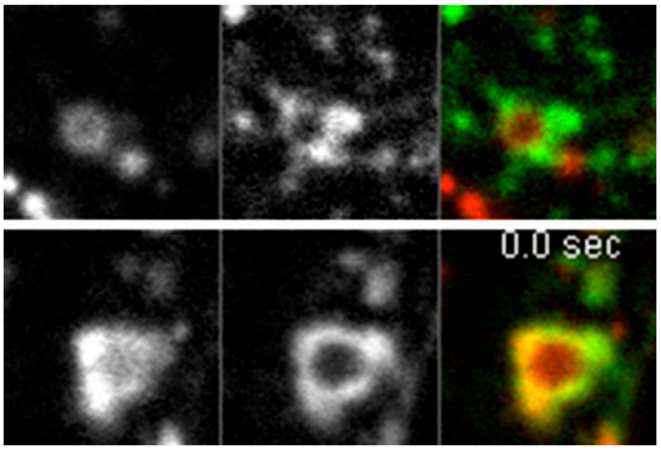
Movie S1. B2AR vesicles are pinched off from tubules marked by cortactin. A typical example endosome containing B2AR (*Left*, red in merged image on *Right*) and cortactin (*Center*, green in merged image on *Right*), imaged every 300 ms, showing a B2AR tubule marked by cortactin, which extends and pinches off a tubule.

Movie S1



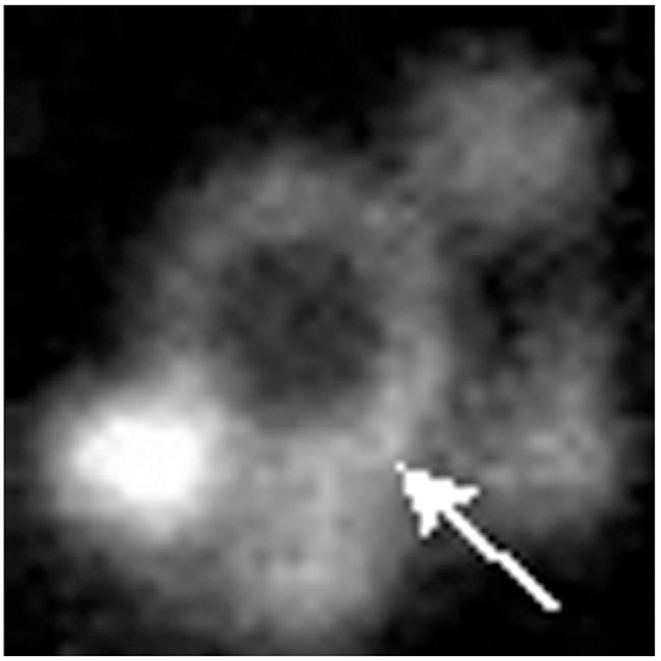
Movie S2. B2ARS>A vesicles are pinched off from multiple tubules, many devoid of cortactin. Shown is a typical endosome containing B2ARS>A (*Left*, red in merged image on *Right*) and cortactin (*Center*, green in merged image on *Right*), imaged every 300 ms. Arrows indicate B2ARS>A tubules pinching off endosomal domains devoid of cortactin.

Movie S2



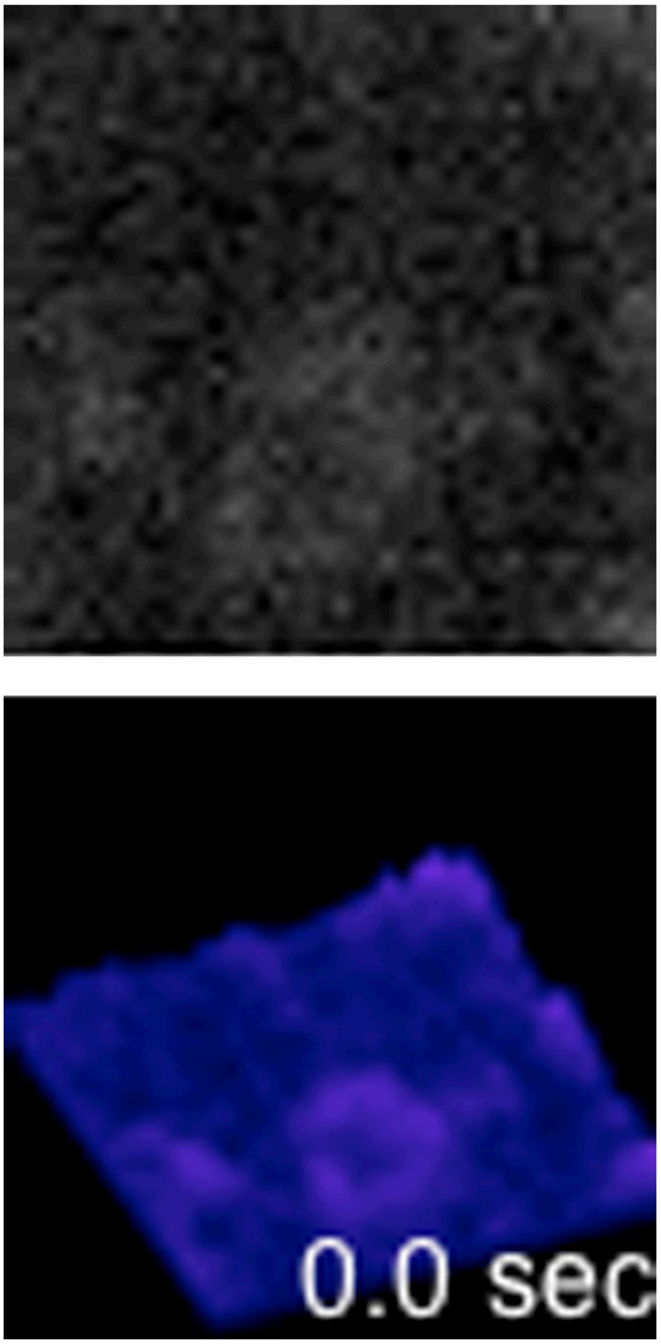
Movie S3. B2AR, but not B2ARS>A, is restricted from entering a subset of tubules that denote bulk recycling tubules. Example endosomes from cells co-expressing TfR, a bulk recycling marker, and either B2AR (*Upper*) or B2ARS>A (*Lower*). The B2AR endosome extrudes multiple endosomal tubules containing TfR but devoid of B2AR. In contrast, B2ARS>A enters almost all of the tubules containing TfR.

Movie S3



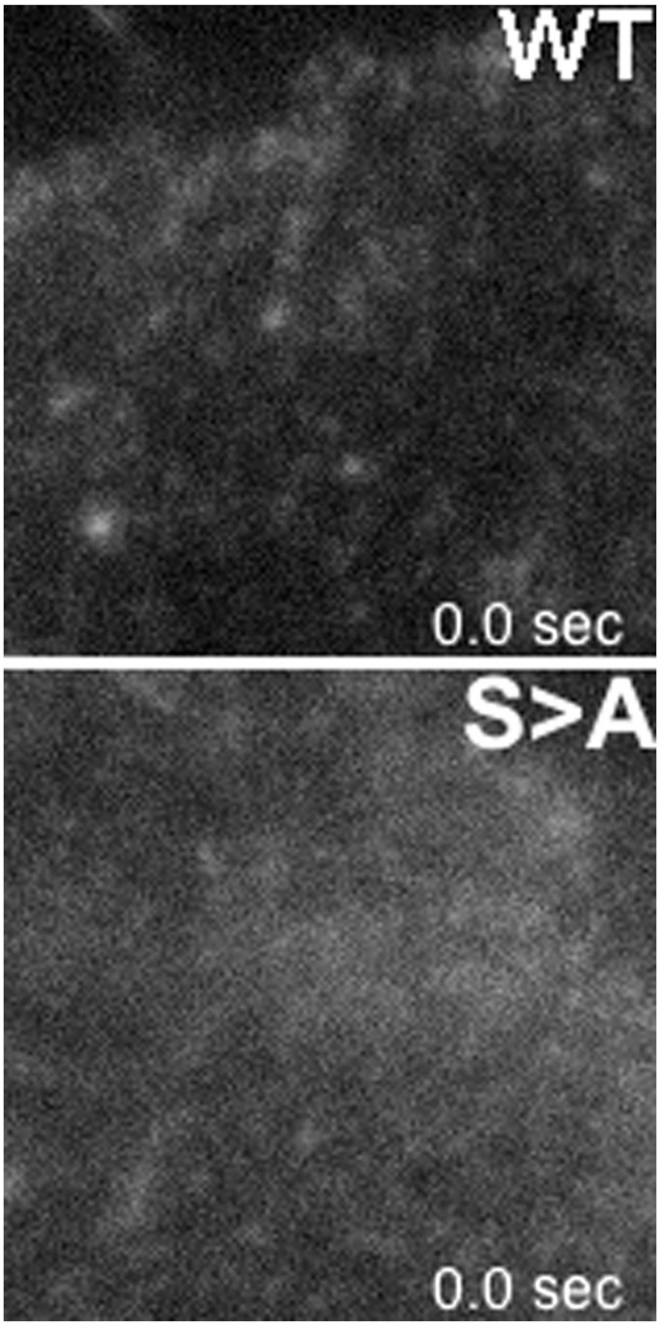
Movie S4. Example fluorescence recovery after photobleaching (FRAP) on a B2ARS>A endosome, imaged at 1 fps. The bleached region is indicated by an arrow. B2ARS>A fluorescence recovered rapidly after photobleaching.

Movie S4



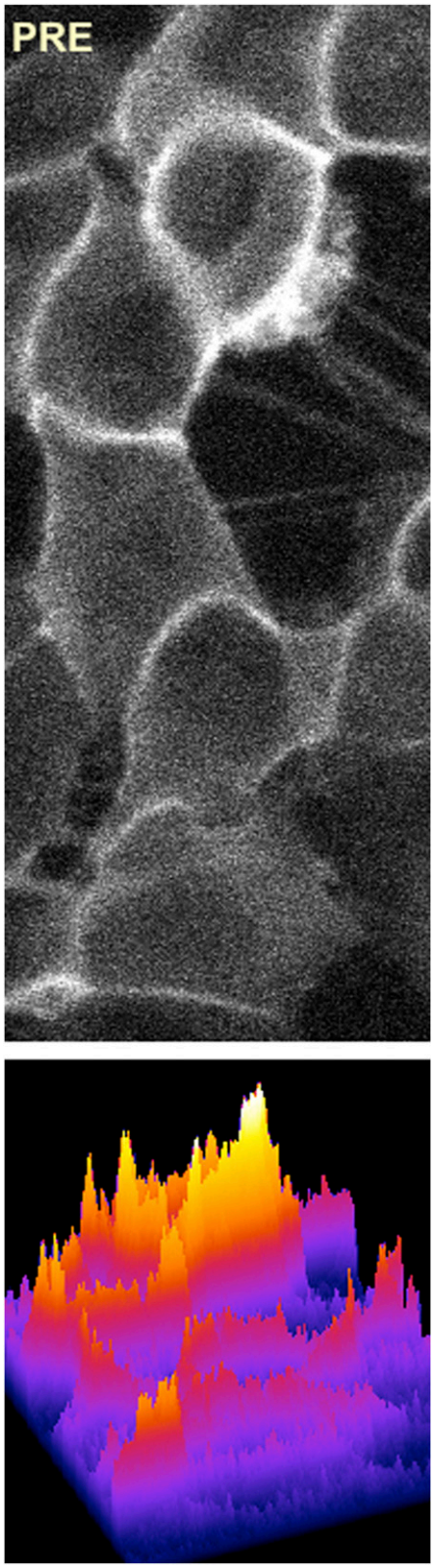
Movie S5. Example of a single B2AR vesicle fusion event. A region of the plasma membrane from a cell expressing SpH-B2AR treated with iso for 5 min, showing two vesicle fusion events mediating B2AR delivery to the cell surface. The heat map surface plot of fluorescence indicates the abrupt spike in fluorescence, followed by isotropic spreading of receptors on the surface.

Movie S5



Movie S6. Actin depolymerization significantly decreases the rate of recycling of B2AR, but not of B2AR S > A. Cells expressing SpH-B2AR or SpH-B2ARS>A were treated with iso for 5 min and then imaged at 10 fps before and after the addition of lat-A. A 30-s segment from each condition is shown. The rate of insertion events decreased rapidly after the addition of lat-A for B2AR, but not for B2ARS>A.

Movie S6



Movie S7. Monitoring B2AR endocytosis in response to isoproterenol and recycling with washout with alp in real time. SpH-B2AR–expressing cells, imaged for a few minutes before (PRE) and for 10 min after (ISO) iso treatment, show rapid loss of fluorescence, indicating removal of surface B2AR by endocytosis. Replacing iso with medium containing alp induces recovery of surface fluorescence by recycling.

Movie S7