Single-molecule diffusion-based estimation of ligand effects on G protein–coupled receptors

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G protein–coupled receptors (GPCRs) are major drug targets. Developing a method to assess the activities of GPCRs is essential for pharmacology and drug screening. However, it is difficult to measure the effects of a drug by monitoring the receptor on the cell surface; thus, changes in the concentrations of downstream signaling molecules, which depend on the signaling pathway selectivity of the receptor, are often used as an index of receptor activity. We show that single-molecule imaging analysis provides an alternative method for assessing the effects of ligands on GPCRs. Using total internal reflection fluorescence microscopy (TIRFM), we monitored the dynamics of the diffusion of metabotropic glutamate receptor 3 (mGluR3), a class C GPCR, under various ligand conditions. Our single-molecule tracking analysis demonstrated that increases and decreases in the average diffusion coefficient of mGluR3 quantitatively reflected the ligand–dependent inactivation and activation of receptors, respectively. Through experiments with inhibitors and dual-color single-molecule imaging analysis, we found that the diffusion of receptor molecules was altered by common physiological events associated with GPCRs, including G protein binding, and receptor accumulation in clathrin-coated pits. We also confirmed that agonist also decreased the average diffusion coefficient for class A and B GPCRs, demonstrating that this parameter is a good index for estimating ligand effects on many GPCRs regardless of their phylogenetic groups, the chemical properties of the ligands, or G protein–coupling selectivity.

INTRODUCTION

Heterotrimetric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs) constitute the largest superfamily of human membrane proteins and are classified into several families based on their sequence similarity (1, 2). About 33% of all small-molecule drugs target just 6% of the ~800 human GPCRs (3, 4); thus, GPCRs have immense potential for drug discovery. However, it is difficult to measure the effects of a drug by monitoring the receptor on the cell surface; thus, changes in the concentrations of downstream signaling molecules, including second messengers, are monitored as an index of receptor activity (5). These conventional methods require background knowledge about the signaling pathways, including coupling specificity to G protein subtypes. Here, we developed an alternative method for assessing ligand effects on GPCRs using microscopy to monitor the movements of receptor molecules in live cells.

Total internal reflection fluorescence microscopy (TIRFM) is a common imaging method for observing single molecules on the bottom plasma membrane of a live cell (6–8). The dimerization and diffusion of the M1 muscarinic receptor (9) and the N-formyl peptide receptor (10) have been measured by TIRFM, but these studies provided limited information about the activation process because the fluorescent dyes were conjugated to agonists, preventing observation of the receptors in their inactive state. Other studies reported that the oligomerization and diffusion of the adrenergic receptors (11, 12), 3-aminobutyric acid type B (GABA_B) receptors (11), and dopamine D2 receptors (13) change upon ligand stimulation; however, the physiological background and generality of these observations are unknown.

Here, we examined the relationship between the diffusion and functional states of metabotropic glutamate receptor 3 (mGluR3) as a model class C GPCR. Class C GPCRs have a large extracellular ligand-binding domain (ECD) on the N-terminal side of the seven α-helical transmembrane domains (TMDs) (Fig. 1A). The ECDs function as an obligate dimer, where dimeric reorientation occurs upon ligand binding (14, 15). The conformational change in ECDs promotes the dimeric rearrangement of TMDs (16–18), activating a protomer of the TMD dimer (19, 20). Single-molecule tracking (SMT) analysis demonstrated that the average diffusion coefficient (Dav) of mGluR3 quantitatively reflected receptor activity. Agonists and inverse agonists increased diffusivity and activation of receptor molecules, respectively, in a dose-dependent manner. Experiments with inhibitors and dual-color TIRFM analysis indicated that the slowing of mGluR3 was related to the decoupling of the receptor–G protein complex, which was followed by receptor accumulation in clathrin-coated pits (CCPs). We verified the generality of this agonist-induced change in the diffusion dynamics of GPCRs by comparing the Dav values of nine GPCRs in various phylogenetic positions. The present findings will contribute to future drug discovery because the estimation of a drug effect on a GPCR from a common change in diffusion dynamics could be used for orphan GPCRs even without knowledge of their downstream effects.

RESULTS

Expression, fluorescent labeling, and single-molecule imaging of HaloTag-fused mGluR3

To determine the relationship between the diffusion coefficient and activity of mGluR3, we monitored the single-molecule movement of...
Fig. 1. Activation model, TIRFM image, MSD-Δt plots, and comparison of diffusion, ligand occupancy, and G protein activation of mGluR3. (A) Activation model of mGluR. The crystal structures of the ECDs of mGluR1 in the inactive (blue; Protein Data Bank (PDB), 1EWT) and active (red; PDB, 1EWK) states were constructed with PyMOL (www.pymol.org). The crystal structure of the TMD (blue and red; PDB, 4OR2) is also shown. (B) Representative TIRFM image of a human embryonic kidney (HEK) 293 cell expressing TMR-labeled mGluR3 (left, whole-cell image; right, magnified view of the area within the blue dashed square). Right: The trajectories of mGluR3 molecules are shown as yellow lines. (C to E) MSD-Δt plots of the trajectories of mGluR3 under the indicated ligand conditions. (C) Inverse agonist (LY341495) dependency. (D and E) Agonist (LY379268) dependencies at 100 nM LY341495 without (D) and with (E) 1 μM MNI137. Data are means ± SEM of 20 cells. *P < 0.01 (two-tailed t test) when compared with the leftmost point in each curve in (J). **P < 0.01 (two-tailed t test) when compared with and without 1 μM MNI137 in (G). (H and I) Dose-dependent changes in specific [3H]LY341495 binding. [3H]LY341495 saturation binding (H, blue squares; dissociation constant (K_d) = 47.4 ± 1.7 nM). Replacement of 100 nM [3H]LY341495 with LY379268 in the absence (H and I, red circles; IC_50 = 0.55 ± 0.08 μM) and presence (I, green squares; IC_50 = 0.60 ± 0.03 μM) of 1 μM MNI137. The amount of nonspecifically bound 100 nM [3H]LY341495 was 50 ± 11 fmol. Data are means ± SEM of three independent experiments. The same membrane preparation from mGluR3-expressing cells was analyzed within the same panel. *P < 0.01 (two-tailed t test) when compared with the leftmost point in each curve in (H). No statistically significant difference was detected with and without 1 μM MNI137 in (I), P > 0.05 by two-tailed t test. (J and K) Dose-dependent changes in the efficiency of G protein activation in membranes from mGluR3-expressing cells. LY341495 dependency (blue closed squares; IC_50 = 2.11 ± 0.18 nM) and LY379268 dependency (red closed circles; EC_50 = 0.025 ± 0.0029 μM) without other ligands are shown in (J). LY379268 dependencies at 100 nM LY341495 without (red closed circles; EC_50 = 1.77 ± 0.39 μM) and with (green closed squares; EC_50 = 9.34 ± 4.44 μM) 1 μM MNI137 are shown in (K). Open circles and squares indicate the G protein activation efficiency of mock-transfected cell membranes under the same ligand conditions as for the closed circles and squares, respectively. Data are means ± SEM of three to five independent experiments. *P < 0.01, **P < 0.03 (two-tailed t test) when compared with the leftmost point in each curve in (J). **P < 0.01 (two-tailed t test) with and without 1 μM MNI137 in (K).
a tetramethylrhodamine (TMR)–labeled HaloTag-fused mGluR3 on HEK 293 cells under various ligand conditions (Fig. 1 and movie S1). The fusion of HaloTag to the C terminus of mGluR3 did not alter receptor dimerization, ligand binding, or G protein activation (fig. S1). In previous studies, N-terminally SNAP-tagged GPCRs were labeled with nonmembrane-penetrable fluorophores (11–13). In this method, only the receptor molecules on the cell surface are labeled at a certain time point, similar to what occurs in a pulse-chase experiment. Therefore, the exocytosis and endocytosis of the receptor molecules after labeling alter the composition of visible receptor molecules on the cell surface depending on the incubation time. In particular, the agonist-induced internalization of the receptors causes selective loss of the activated receptors. In contrast, we used membrane-penetrable fluorophores, which enabled uniform labeling of the receptor molecules in a cell. Whole-cell labeling is important for monitoring the total number of receptor molecules, with and without ligand, on the cell surface, including newly exocytosed receptors after labeling.

When using membrane-penetrable fluorophores, nonspecific binding of the fluorophores in the cell should be evaluated carefully. We used the HaloTag TMR ligand (TMR), HaloTag STELLA Fluor 650 ligand (SF650), and the SNAP-Cell 647–SiR ligand (SiR) to label GPCRs or G proteins. TMR showed the least nonspecific binding in mock-transfected cells (fig. S2, A to F). It was difficult to use a concentration of SF650 or SiR >300 nM for single-molecule imaging because of the high amount of nonspecific binding (fig. S2, D to F). We also evaluated the specific binding affinity of fluorophores to Halo- or SNAP-tagged proteins based on the difference in fluorescence intensity between cells expressing the tagged protein and those cells that did not express the tagged protein (fig. S2, A to C). The affinity for the HaloTag was fourfold greater for SF650 than for TMR. In terms of photostability and brightness, SF650 also performed better than TMR under our experimental conditions (fig. S2, G and H, and see Materials and Methods). However, TMR achieved a higher rate of dye labeling under low nonspecific binding conditions. In the single-molecule imaging of mGluR3, ~95% of the HaloTag-fused mGluR3 molecules were specifically labeled with 300 nM TMR, according to the saturation binding assay using the HaloTag TMR ligand (fig. S2A and Materials and Methods). Because HEK 293 cells express no detectable mRNA for mGluRs (21), almost all receptor molecules were labeled in the current measurements. The mean density of receptor molecules on the cell surface was 0.40 ± 0.11 particles/μm² in the single-molecule images (fig. S2I).

**MSD-Δt analysis of HaloTag-fused mGluR3**

Time-dependent mean square displacement (MSD-Δt) plot analysis is often used to evaluate the diffusion coefficient and diffusion mode of membrane proteins (7, 9, 11, 13). The MSD corresponds to the average squared distance that a receptor molecule travels from its starting point within a certain time interval, Δt, which is proportional to the apparent lateral diffusion coefficient. A linear MSD-Δt plot is observed when the receptor molecules exhibit simple Brownian diffusion (22). On the other hand, a concave up- or down-shaped MSD-Δt plot suggests that the receptor molecules exhibit directed or confined diffusion modes, respectively (22).

We quantified the MSD from the trajectories traced by SMT in each cell (Fig. 1B) and analyzed the dose-dependent change in the total average MSDs of the trajectories (Fig. 1, C to E). Stimulation of cells with the inverse agonist LY341495 statistically significantly increased the MSD of mGluR3 molecules (Fig. 1C). In contrast, stimulation with the agonist LY379268 statistically significantly decreased the MSD in a dose-dependent manner (Fig. 1D). We also analyzed the LY379268-dependent change in the diffusion of mGluR3 in the presence of 1 μM MNI137, a negative allosteric modulator (NAM) (23). MNI137 binding to the TMD suppressed the agonist-dependent decrease in the MSD, and no statistically significant difference was observed upon stimulation with LY379268 (Fig. 1E).

**Relationship among the average diffusion coefficient, ligand-binding affinity, and G protein activation efficiency of mGluR3**

We calculated the $D_{Av}$ of mGluR3 molecules from the MSD values (Eqs. 1 and 2 in Materials and Methods). The dose-dependent curves showed the LY341495-induced increase and LY379268-induced decrease in $D_{Av}$ in the absence of other ligands (Fig. 1F). The LY379268-dependent decrease in $D_{Av}$ was greater in the presence of 100 nM LY341495 and was statistically significantly suppressed by the addition of 1 μM MNI137 (Fig. 1G). To compare the dose dependency of $D_{Av}$ with the ligand-binding affinity of mGluR3, we performed an in vitro $[^{35}S]$GTPγS binding assay (Fig. 1, H and I). The half-maximal effective concentration (EC50) value of the LY341495-induced increase in $D_{Av}$ (Fig. 1F) was at most half that of $[^{35}S]$GTPγS binding (Fig. 1H). The half-maximal inhibitory concentration (IC50) values of the LY379268-induced decrease in $D_{Av}$, without and with 100 nM LY341495 (Fig. 1, F and G) were at most twice that of the competition binding curve between LY379268 and 100 nM $[^{35}S]$GTPγS (Fig. 1H). These results suggest that the dose dependency of $D_{Av}$ corresponded well with the ligand-binding affinity. The effect of MNI137 on mGluR3 could not be measured by the $[^{35}S]$GTPγS binding assay; no statistically significant difference was observed with and without 1 μM MNI137 (Fig. 1I).

We also measured the G protein activation efficiencies of mGluR3 under the same ligand conditions with an in vitro $[^{35}S]$GTPγS binding assay. We found that mGluR3 showed high G protein activation even without ligands, and this basal activity was suppressed by LY341495 in a concentration-dependent manner (Fig. 1J). This is consistent with a study demonstrating that Cl− binding to the ECD causes high basal activity of mGluR3 (24, 25). Thus, the inverse agonist–induced increase and agonist–induced decrease in $D_{Av}$ (Fig. 1F) likely reflect the change in the equilibrium between the inactive and active states of mGluR3 molecules on the cell surface. Furthermore, 1 μM MNI137 statistically significantly suppressed the agonist-induced increase in G protein activation efficiency (Fig. 1K), as was expected from the $D_{Av}$ of mGluR3 (Fig. 1G).

The IC50 of the LY341495-induced suppression of the basal activity (Fig. 1F) was one order of magnitude less than those obtained from the ligand-binding assay (Fig. 1H) and from the dose dependency of $D_{Av}$ (Fig. 1F). Furthermore, there was a difference of two orders of magnitude between the EC50 values of the LY379268-dependent increase in G protein activation efficiencies with (Fig. 1K) and without (Fig. 1J) 100 nM LY341495, whereas that with 100 nM LY341495 was similar to those estimated from the ligand-binding assay (Fig. 1H) and from the dose dependency of $D_{Av}$ (Fig. 1, F and G). Generally, it is difficult to estimate the ligand occupancy from a downstream response after amplification of the signaling cascade because the response is usually saturated at a ligand concentration that is less than the saturation binding (26). Single-molecule imaging analysis enabled us to assess the fraction of receptors in the inactive and active states, which corresponded well to the fraction of ligand binding.
Ligand-induced changes in the mGluR3 diffusion state distribution

Next, we performed variational Bayesian-hidden Markov model (VB-HMM) clustering analysis (27, 28) to classify the diffusion states of mGluR3. VB-HMM analysis of the total trajectories suggested that the diffusion of mGluR3 molecules could be classified into four states (immobile, slow, medium, and fast; Fig. 2, A and B, fig. S3, and movie S1). The fast and medium states contained transient directional and non-directional movements, and their MSD-Δt plots were linear on average (fig. S3, C and D). In contrast, concave, down-shaped MSD-Δt plots were observed for the slow and immobile states (fig. S3, E and F), indicating the confined diffusion of mGluR3 (22). The confinement lengths were estimated to be 140 and 70 nm, respectively (fig. S3, E and F, and Materials and Methods), which were consistent with the radii of plasma membrane microdomains (29). The distribution of the apparent oligomer size of mGluR3 in each diffusion state was estimated from the intensity histogram based on the sum of Gaussian functions. The mean intensity of monomeric TMR estimated from the intensity histogram of TMR-labeled CD86, a monomeric membrane protein on HEK 293 cells (fig. S3G) (11), was about half that of the highest peak in the histogram of mGluR, suggesting that most of mGluR forms dimers (fig. S3H).

The higher-order clusters of mGluR3 were mainly related to the immobile state, where the intensity histogram was right-shifted compared with the other diffusion states (fig. S3H).

Upon stimulation with the inverse agonist LY341495, the fraction of molecules in the fast state was statistically significantly increased, whereas the fractions of immobile and slow state molecules decreased in a dose-dependent manner (Fig. 2C). In contrast, stimulation with the agonist LY379268 increased the fraction of molecules in the immobile and slow states but decreased the fraction in the fast state (Fig. 2D). To analyze the transitions among the four states, we estimated the time constants of the state transition from the VB-HMM transition array (Fig. 2E and fig. S4). The dose-dependent changes were mainly observed in the transition from the slower to the faster states, suggesting that the activation of mGluR3 made it difficult to escape from the microdomain and that mGluR3 was trapped in a slower state. The diffusion coefficients of the medium and slow states estimated from the VB-HMM analysis were also statistically significantly changed upon ligand stimulation (Fig. 2E and fig. S5). The ligand-induced changes in $D_m$ (Fig. 1, F and G) were derived from the opposing change in the fraction of molecules in the fast state compared with those in the slow and immobile states and also from the changes in the diffusion coefficients of molecules in the medium and slow states (Fig. 2E).

**Fig. 2.** VB-HMM analysis of the trajectories of mGluR3 molecules. (A) Every step in the trajectories shown in Fig. 1B was categorized into four diffusion states. The immobile, slow, medium, and fast states are shown in blue, yellow, green, and red, respectively. (B) Histogram of the displacement during 30.5 ms of all of the trajectories (open black bars; 28,092 steps from 573 trajectories) on a cell divided into four single-step distributions of random walks (see Eq. 6 in Materials and Methods). The immobile, slow, medium, and fast states are shown in blue, yellow, green, and red, respectively. (C and D) Dose-dependent changes in the fractions of the diffusion states. (C) LY341495 dependency. (D) LY379268 dependencies in the presence of 100 nM LY314195. The immobile, slow, medium, and fast states are shown in blue, yellow, green, and red, respectively. All data are means ± SEM of 20 cells. *$p < 0.01$ (two-tailed t test) when compared with the leftmost point in each curve. (E) Four state transition diagrams of mGluR3 under inactive (1 µM LY314195) and active (100 µM LY379268 with 100 nM LY341495) ligand conditions. The diffusion coefficient and fraction of each state are shown next to the circles, the size of which reflects the size of the fraction. The SEM is indicated in parentheses ($n = 20$ cells). The arrows between states reflect the rate constants of the state transition estimated from fig. S4. The statistically significant changes in rate constants between the two conditions in fig. S4 are shown as colored arrows (blue, inactive; red, active). The asterisk indicates statistically significant differences in the fractions or in the diffusion coefficients compared with the inactive ligand conditions ($^{*}p < 0.01$ by two-tailed t test).
Effects of pertussis toxin on mGluR3 molecule diffusion

We analyzed the effect of pertussis toxin (PTX), an inhibitor of \( G_{\text{i/o}} \) protein activation by blocking the GPCR–G protein interaction, to link the diffusion state with the G protein–bound state of mGluR3 (Fig. 3A). Treatment with PTX resulted in a statistically significant decrease in the \( D_{\text{Av}} \) (Fig. 3B to D, top), reflecting a decrease in the fraction of mGluR3 molecules in the fast state and an increase in the fraction of molecules in the immobile state under basal (vehicle), inactive (100 nM LY341495), and active (100 \( \mu \)M LY379268) conditions, respectively (Fig. 3B to D, bottom). To confirm that the effect of PTX was caused by loss of the interaction between mGluR3 and \( G_{\text{i/o}} \), we analyzed the effects of the B oligomer of PTX as a negative control. The B oligomer carries the A protomer that catalyzes adenosine 5'-diphosphate (ADP) ribosylation of the \( G_{\text{i/o}} \alpha \) subunit (Fig. 3A) (30). Treatment of cells with the PTX B oligomer alone did not alter the diffusion of mGluR3 (Fig. 3B to D, middle), indicating that ADP ribosylation of the \( G_{\text{i/o}} \alpha \) subunit by the PTX A protomer was responsible for slowing the diffusion of mGluR3. These results suggest that the fast diffusion state contained \( G_{\text{i/o}} \) protein–bound mGluR3 molecules under both the inactive and active ligand conditions. This is consistent with previous studies, which demonstrated precoupling of GPCRs with G protein even in the inactive state, enabling fast signal transduction.
The activation of mGluR3 triggered a release of $G_{i/o}$ from the precoupling complex, similar to PTX-induced decoupling (Fig. 3A), thereby decreasing the fraction of mGluR3 molecules in the fast state. Thus, the decrease in $\Delta t_{av}$ upon agonist stimulation (Fig. 1, F and G) may be partly explained by the decrease in mGluR3 coupling with $G_{i/o}$ proteins.

**Dual-color TIRFM analysis of mGluR3 and $G_o$ protein colocalization**

To observe the interaction between mGluR3 and G protein directly, we performed dual-color single-molecule imaging analysis. We detected the colocalization of TMR-tagged mGluR3 and SiR-tagged $G_o$ protein on HEK 293 cell membranes in the presence of 1 μM LY341495 (inactive) or 100 μM LY379268 (active) with or without PTX treatment. The rate of SiR dye labeling of $G_o$ protein was estimated as ~12% (Fig. S2C). We observed mGluR3 molecules that colocalized with $G_o$ proteins under both the inactive and the active ligand conditions (Fig. 4, A and B, and movie S2). The interaction with the $G_o$ protein occasionally accelerated mGluR3 diffusion (movie S2). PTX treatment statistically significantly decreased the MSD of mGluR3 in the presence of exogenous $G_o$ protein at saturating ligand concentrations (Fig. 4, C

Fig. 4. Colocalization analysis of mGluR3 and $G_o$ protein. (A and B) Representative images of colocalization between TMR-labeled mGluR3 (red) and SiR-labeled $G_o$ protein (green). The mGluR3 and $G_o$ proteins formed transient complexes in both the inactive (A; 1 μM LY341495) and active (B; 100 μM LY379268) conditions. Scale bars, 1 μm. Images are representative of 20 cells. (C and D) Comparison of the MSD-$\Delta t$ plots of the trajectories of mGluR3 in dual-color TIRFM movies with and without PTX. The MSD-$\Delta t$ plots for the inactive (1 μM LY314195) and active (100 μM LY379268) ligand conditions are shown in (C) and (D), respectively. (E) The fraction of mGluR3 molecules that colocalized with $G_o$ proteins was estimated from the total trajectories under the inactive (blue) and active (red) ligand conditions with and without PTX. (F) Cumulative probability histograms of the colocalization duration under the inactive (blue) and active (red) ligand conditions without (filled markers) and with (empty markers) PTX. Curves were fitted using a two-component exponential function (see Eq. 8 in Materials and Methods). (G and H) Comparison of the fractions of the diffusion states estimated from VB-HMM analysis without (G) and with (H) PTX. The fractions estimated from the trajectories of total mGluR3 molecules under the inactive and active ligand conditions are indicated by the blue and red shaded bars, respectively. The fractions estimated from the trajectories of mGluR3 colocalized with $G_o$ protein under the inactive and active ligand conditions are indicated by blue and red solid bars, respectively. Data in (C) to (H) are means ± SEM of 20 cells. *P < 0.01 and #P < 0.05 by two-tailed t test.
and D). The ligand conditions did not affect the probability of mGluR3 and G<sub>i</sub> protein colocalization; however, PTX treatment statistically significantly decreased it (Fig. 4E). These decreases were caused mainly by the decreased on-rate between mGluR3 and G<sub>i</sub> proteins, because no statistically significant difference was observed in the cumulative histogram of colocalization duration (Fig. 4F). We compared the diffusion state fraction of mGluR3 colocalized with G<sub>i</sub> protein (mGluR3/G<sub>i</sub>) with that of mGluR3 colocalized with total mGluR3 (mGluR3/total) in the presence or absence of PTX. Under all conditions, the fractions of the fast and medium states were statistically significantly greater for mGluR3/G<sub>i</sub> than for mGluR3/total (Fig. 4, G and H). Together, these data suggest that the PTX-induced deceleration of mGluR3 movement is explained mainly by inhibited formation of the mGluR3–G protein complexes, which diffuse faster than mGluR3 alone.

**Dual-color TIRFM analysis of mGluR3 colocalization with clathrin**

We investigated the physiological events related to the immobile and slow states that increased upon activation of mGluR3, in contrast to the decrease in the fraction of mGluR3 molecules in the fast state. A TIRFM image showed that the immobile state was related to the clustering of mGluR3 molecules, which was followed by internalization (Fig. 5A). Immobile clusters of mGluR3 were formed and disappeared with rapid directional movement (movie S3). To test whether the clusters were receptors in CCPs, we analyzed the colocalization of TMR-labeled mGluR3 and green fluorescent protein (GFP)–labeled clathrin light chain (CLC) by dual-color TIRFM. When mGluR3 and CLC were colocalized, TMR intensity increased rapidly (Fig. 5, B and C, and movie S4). The intensities of TMR and GFP decreased simultaneously several seconds after colocalization (Fig. 5C). These results suggest that mGluR3 formed a large cluster in a CCP and that the cluster was internalized as a clathrin-coated vesicle in the cytoplasmic region, which could not be reached by the evanescent light (Fig. 5A).

Next, we quantified the distribution of the diffusion states of mGluR3 colocalized with CLC (mGluR3/CLC) and compared it with the total number of mGluR3 molecules (mGluR3/total) for the inactive (100 nM LY341495) and active (100 μM LY379268) ligand conditions. The immobile and slow state fractions for mGluR3/CLC were statistically significantly increased compared to those for mGluR3/total, suggesting that clathrin binding immobilized the receptor (Fig. 5D, blue and red lines). Comparing the inactive and active ligand conditions demonstrated that the fraction of the immobile state of mGluR3/CLC increased upon activation (Fig. 5D, black lines). Furthermore, the probability and time constant of the colocalization between mGluR3 and CLC were statistically significantly increased after activation (Fig. 5, E to G). The cumulative histogram of the colocalization duration was fitted with a double exponential function with short and long time constants (Fig. 5F). The time constants were ~1.5 times greater for the active than for the inactive ligand condition (Fig. 5G), indicating that the ~1.8-fold increase in the probability of colocalization observed (Fig. 5E) was caused mainly by the increased duration of colocalization. Thus, the immobile state fraction in the total trajectories reflected the number of mGluR3 molecules interacting with clathrin molecules, which increased upon activation.

**Effects of RNA interference–mediated CLC inhibition on mGluR3 molecule diffusion**

To distinguish between mGluR3 activation and internalization, we measured the effects of RNA interference (RNAi)–mediated CLC knockdown on mGluR3 diffusion (Fig. 6). Western blotting analysis indicated that transfection of HEK 293 cells with small interfering RNAs (siRNAs) specific for CLC reduced the abundance of CLC by ~50% (Fig. 6A). Knockdown of CLC statistically significantly increased the average MSD of mGluR3 under the basal (vehicle) and active (100 μM LY379268) ligand conditions (Fig. 6, B and D), reflecting decreases in the fraction of receptors in the slow and immobile states and an increase in the fraction of receptors in the fast state (Fig. 6, E and G). In contrast, no statistically significant changes were observed under the inactive (1 μM LY341495) ligand condition (Fig. 6, C and F). These results suggest that RNAi-mediated knockdown of CLC decreased the amount of slowly diffusing mGluR3–clathrin complex and increased the fraction of the fast state including mGluR3–G protein complex.

**Correlation between receptor density, mean oligomer size, and D<sub>Av</sub>**

We also analyzed the ligand-induced changes in mean oligomer size, which should be related to internalization. However, the mean oligomer size showed no clear dose dependency (fig. S6, A to C). This may be due to the higher correlation between mean oligomer size and receptor density (fig. S6, D to G). The mean oligomer size of mGluR3 was statistically significantly and positively correlated with receptor density (fig. S6G). Thus, the strict selection of cells based on receptor density is required to test the ligand effect on oligomer size. In contrast, no statistically significant correlation was observed between receptor density and D<sub>Av</sub> (fig. S7). Thus, these data suggest that D<sub>Av</sub> is a robust index of mGluR3 activity that is independent of receptor abundance.

**Generality of the agonist-induced changes in GPCR diffusion**

To test the generality of the relationship between the diffusion and activation of GPCRs, we monitored the single-molecule movement of fluorescently labeled GPCRs of other classes (Table 1 and movie S5). Because it is not necessary to label all of the molecules in a cell to measure D<sub>Av</sub>, we used 30 nM SF650 for labeling to improve the quality of the single-molecule imaging. Under the labeling conditions, ~70% of receptors were labeled with SF650 with lower nonspecific binding (fig. S2B). We compared the MSD–Δt plots of the trajectories of GPCR molecules with and without agonist stimulation (fig. S8) and calculated the D<sub>Av</sub> values (Table 1). All of the GPCRs tested showed statistically significant slowing upon agonist stimulation regardless of their phylogenetic positions, the chemical properties of the ligands, or their G protein–coupling selectivity (Table 1 and fig. S8). In the absence of ligands, mGluR3 had a reduced D<sub>Av</sub> (0.047 μm<sup>2</sup>/s) compared to those of other GPCRs (0.06 to 0.09 μm<sup>2</sup>/s), which corresponded to that of mGluR3 on cells treated with 1 μM LY341495 (0.064 μm<sup>2</sup>/s; Table 1). Thus, the diffusion coefficient of mGluR3 was similar to those of other GPCRs in the inactive state (Fig. 2E). In the presence of an agonist, the D<sub>Av</sub> of GPCRs was 0.04 to 0.07 μm<sup>2</sup>/s. The effect of a drug on each GPCR was accurately detected by SMT analysis as a change in D<sub>Av</sub> (Table 1), but the absolute values of D<sub>Av</sub> varied between GPCRs.

**DISCUSSION**

Here, we provide a method for assessing the effects of drugs on GPCRs by monitoring the diffusion behavior of GPCRs. We first obtained proof of concept of the applicability of single-molecule imaging to...
the pharmacology of a class C GPCR, mGluR3. The basal activity, agonist-induced activation, inverse agonist–induced inactivation, and NAM-dependent suppression of activity were evaluated by measuring the $D_{AV}$ of mGluR3 on the living cell surface (Fig. 1). The dose-dependent change in $D_{AV}$ was derived from the population shift of the four diffusion states and the change of the diffusion coefficient...
of each state (Fig. 2E). The plasma membrane is partitioned into submicrometer-scale domains with different lipid compositions, including lipid rafts (35). Assuming that the four diffusion states are determined primarily by the lipid environment of the receptor molecules, ligand-induced conformational changes followed by interactions with other molecules would alter the accessibility of the receptor to membrane domains. A previous single-molecule imaging study also suggested that the lateral diffusion of transmembrane proteins is transiently anchored by the actin cytoskeleton, which impedes free diffusion across the membrane domains (36). Furthermore, CCPs on the plasma membrane restrict the receptors to confined areas (37). Our data suggest that treatment with the agonist and inverse agonist increased and decreased, respectively, the distribution of mGluR3 molecules within these microdomains,

![Fig. 6. Effects of the RNAi-mediated knockdown of CLC on the molecular behavior of mGluR3. (A) Western blotting analysis of CLC abundance in HEK 293 cells transfected with mock siRNA (Lipofectamine RNAiMax transfection reagent was used without any siRNA) or CLC-specific siRNA. Top left: The bands observed in the 25- to 30-kDa range in the upper panel represent CLC. Bottom left: Western blotting analysis of β-actin, used as a loading control, in the same lysates. Western blots are representative of four experiments. Right: Comparison of normalized density of Western blots. Data are means ± SEM of four experiments. (B to D) Comparison of the MSD-Δt plots of the trajectories of mGluR3 in cells transfected with mock siRNA or CLC-specific siRNA. MSD-Δt plots are shown for the basal (vehicle) (B), inactive (1 μM LY314195) (C), and active (100 μM LY379268) (D) ligand conditions. (E to G) Comparisons of the fractions of the diffusion states estimated from VB-HMM analysis of the data shown in (B) to (D). Data are means ± SEM of 20 cells per condition. *P < 0.01, **P < 0.05 when compared to Mock as assessed by two-tailed t test.

Table 1. Comparison of the $D_{av}$ values of nine GPCRs in various phylogenetic positions with or without ligands. The class, group, and cluster of GPCRs are listed according to previous reports (1, 2). $D_{av}$ was calculated from the MSD in fig. S8 based on Eqs. 1 and 2 in Materials and Methods. All data are means ± SEM of 20 cells. P values indicating the statistically significant difference between the vehicle and ligand conditions were calculated on the basis of Welch’s two-tailed t test. Under ligand conditions, GPCR-expressing HEK 293 cells were stimulated by the compound listed in the rightmost column. MECA is initials of GPCR in this branch: the melanocortin receptors (MCRs), endothelial differentiation G-protein coupled receptors (EDGRs), cannabinoid receptors (CNRs), and adenosin binding receptors (ADORAs); DHA, docosahexaenoic acid; NECA, 5′-N-ethylcarboxamidoadenosine; TRAP-6, thrombin receptor activator peptide 6.
which was partly related to the binding partner of mGluR3, such as G protein and clathrin (Fig. 7).

The PTX treatment assay suggests that the fast state is related to the binding of mGluR3 to a G protein (Figs. 3 and 4). Before performing the experiment, we expected that PTX would have an effect only under the active ligand conditions; however, this was not the case. The slowing of mGluR3 by PTX was observed more clearly under the inactive ligand conditions (Figs. 3 and 4), providing evidence of mGluR3−G protein precoupling. Furthermore, we directly observed transient formation of the mGluR3−G protein complex under both the inactive and the active ligand conditions (Fig. 4, A and B, and movie S2). PTX treatment inhibited the formation of the mGluR3/Gα complex, which diffused faster than the average mGluR3 molecule (Fig. 4, E, G, and H). Although the fraction of fluorescent Gα-coupled mGluR3 molecules was small (~0.4% of the total mGluR molecules), at least ~3.3% of mGluR3 molecules were colocalized with Gαs considering the dye labeling rate of Gαs (~12%) and the photobleaching of fluorophores during the measurements. Taking into account the endogenous Gα proteins, whose precise concentrations were unknown, the actual fraction of the mGluR3/Gα complex was higher. Thus, the twofold reduction in the mGluR3/Gα protein complex by PTX treatment (Fig. 4E) could explain the ~4% change in the diffusion state distribution of total mGluR3 molecules (Figs. 3 and 4).

Precoupling with G proteins has been demonstrated for class A GPCRs, such as adrenergic and muscarinic acetylcholine receptors. The ternary complex model, which assumes that receptor/G protein precoupling induces the high-affinity state of GPCRs, was proposed before the discovery of G proteins (38), and it is widely accepted to depict the properties of agonist/GPCR associations. Although there is no previous report demonstrating the high-affinity state formation of mGlurRs to our knowledge, a previous study using nanodiscs demonstrated that a single TMD of mGlur2, which cannot bind to glutamate, can couple with G proteins (39). However, the basal activity of GPCRs, including mGlur3, makes it difficult biochemically to distinguish the precoupled receptor−G protein complex from the binding of spontaneously activated receptor to G proteins. Studies using Förster resonance energy transfer and fluorescence recovery after photobleaching confirmed the presence of precoupling complexes of the α2A adrenergic receptor, the muscarinic M3 and M4 receptors, the adenosine A1 receptor, and protease-activated receptor 1 under the inactive ligand conditions (31–34). Here, we could not distinguish the precoupling complex from the active complex by colocalization probability or duration (Fig. 4, E and F). From the view point of diffusion rate, the active complex showed a trend toward becoming immobile compared with the precoupling complex (Fig. 4, G and H). This is consistent with a dual-color TIRFM analysis of adrenergic receptors and G proteins, suggesting that the active receptor−G protein complex is formed in a confined region of the plasma membrane (12). The difference in mobility between precoupling and active complexes may be an additional reason for the increase in the fraction of immobile mGluR3 molecules upon activation.

Currently, it is unknown why G protein binding sometimes accelerates the diffusion of mGluR3 molecules (Fig. 4G and movie S2), although simple physical models predict slower diffusion of particles of larger volume (40). We speculate that the major determinant of the diffusion coefficient of mGluR3 is the viscosity of the membrane surrounding the receptor molecule, which is dependent on the lipid composition. G protein binding would increase the accessibility of mGluR3 to a less viscous membrane environment. In addition, another study revealed that crossing the diffusion barrier was controlled by the interaction between the C-terminal region of mGluR5 and a cytosolic partner in astrocytes (41). Receptor−G protein precoupling, in which the C-terminal region of the GPCR also plays an essential role (33), would affect the ability to cross the membrane microdomain for a similar reason.

The recruitment of GPCRs into CCPs is a well-established mechanism for endocytosis regardless of the GPCR family. Our dual-color TIRFM analysis demonstrated that the immobile state of mGluR3 was related to its interaction with clathrin molecules (Fig. 5). During desensitization, GPCRs are phosphorylated by GPCR kinases, which is followed by the recruitment of arrestins (42). The GPCR-arrestin complexes are then gathered into CCPs through interactions between arrestin, clathrin, and the AP2 adaptor (42). Previously, the clathrin-mediated endocytosis of class A GPCRs, including adrenergic and opioid receptors, was analyzed by TIRFM under high-expression conditions in which a single receptor molecule could not be resolved, and it was demonstrated that the GPCR cargo regulates the surface residence time of CCPs through the PDZ-mediated interaction of the GPCR with the actin cytoskeleton and through receptor ubiquitylation (43, 44). Our results suggest that the time constant of colocalization between mGluR3 and CLC molecules increased upon agonist stimulation (Fig. 5, F and G), which is qualitatively consistent with the previous reports. The absolute values of the colocalization time constant were two orders of magnitude less than the previously reported values for bulk imaging, and this is probably due to the higher photobleaching rate of single TMR ligands (~3 s) in single-molecule imaging. Thus, it is rare to observe the whole process, from the recruitment of receptors into the CCP to the internalization, as we showed (Fig. 5, B and C), where the clustering rate of the receptor-clathrin complex was greater than the photobleaching rate. Experiments involving RNAi-mediated knockdown of CLC also suggested that mGluR3 molecules in slow and immobile states interacted with clathrin (Fig. 6). These results are consistent with a single-molecule imaging analysis of neurokinin-1 receptor, a class A GPCR (37).

These physiological events, which affect diffusion, are not specific to class C GPCRs. If the effect of a drug on a GPCR can be estimated from a common change in the diffusion dynamics, we could perform drug
assessments of GPCRs without knowing the specific signaling cascades stimulated by the receptors. Therefore, we verified the generality of the activation-induced change in receptor diffusion for various GPCRs of other classes. Comparison of the diffusion coefficients of GPCRs with and without an agonist demonstrated that the slowing of receptor diffusion upon activation by an agonist is a general feature of GPCRs irrespective of the signaling pathways downstream of the receptor (Table 1 and fig. S8). The agonist-induced increases in the diffusion coefficients of GABA<sub>B</sub> (11) and dopamine D<sub>2</sub> receptors (13) were reported in previous studies. The apparent discrepancy between the present study and previous studies is attributed to the labeling method used in each study, as described earlier. Furthermore, there is a clear difference between the analysis in the present study and that used in the study by Tabor et al. (13), who excluded slow-moving receptors (<0.02 μm<sup>2</sup>/s). If we performed a similar analysis here, the immobile and slow diffusion fractions of mGluR3 would be almost completely filtered out, resulting in a misleading evaluation of <i>D<sub>AV</sub></i>.

In conclusion, our data suggest that the diffusion coefficient is a good index for estimating the effects of a drug on various GPCRs on a living cell. The present method can be applied to HEK 293 cells transiently expressing fluorescently labeled GPCRs because <i>D<sub>AV</sub></i> is mostly unaltered by variability in the cell surface receptor density (fig. S7). On the other hand, the mean oligomer size was positively correlated with the receptor density on the cell surface (fig. S6). Further comprehensive dual-color TIRFM analyses are required to assess the broad generality of the state-dependent dimerization or oligomerization of GPCRs. Because it is possible to quantify the diffusion of any GPCR by TIRFM, our technique could be useful for drug screening of many GPCRs, including orphan GPCRs, about which little is known. We anticipate that the present study will contribute to the future development of a single-molecule dynamics-based pharmacological method for GPCRs.

**MATERIALS AND METHODS**

**Materials**

[35S]<sup>g</sup>GTP<sub>S</sub> (37 TBq/mmol) was purchased from PerkinElmer Life Sciences. PTX and B oligomer were purchased from Wako Chemicals. [3H]LY341495 [1.28 terabecquerel (TBq)/mmol], LY341495, LY379268, MNI137, NECA, and serotonin were purchased from Tocris Cookson. Human CD86 complementary DNA (cDNA) was purchased from OriGene. The siRNAs targeting CLCs (5′-GGCUUAAAGGGUGUGUUGUUG-3′) were amplified by PCR and inserted into the loop encoding mGluR3. After overnight incubation, the HaloTag-fused mGluR3 was labeled with 300 nM HaloTag TMR ligand (Promega) in a 60-mm dish for 15 min at 37°C under 5% CO<sub>2</sub>. For the inhibitor assay, the cells were treated with 5 nM PTX, 5 nM B oligomer, or vehicle for 6 hours at 37°C under 5% CO<sub>2</sub> before imaging. Cells were washed twice with phosphate-buffered saline (PBS) and DMEM/F12 without FBS before being treated with the HaloTag ligand. The coverslip was mounted on a metal chamber (Invitrogen) and then washed five times with Hanks’ balanced salt solution (HBSS) (400 μl; Sigma-Aldrich), with 15 mM Hepes (pH 7.3) and 0.01% bovine serum albumin (BSA), without NaHCO<sub>3</sub>. Ligand (5× concentration) or vehicle solution (100 μl) was added to the chamber with 0.01% BSA/HBSS (400 μl) 10 min before imaging. Single-molecule imaging was performed 10 to 30 min after the addition of ligand (or vehicle) at room temperature (25°C). The fluorescently labeled GPCRs on the basal cell membrane were observed by total internal reflection illumination with an inverted fluorescence microscope (TE2000, Nikon). The cells were illuminated with a 559-nm, 50-mW laser (WS-0559-050, NTT Electronics) or a 532-nm, 100-mW laser (Compass 315M-100) with an ND50 filter for fluorescence microscopy. The cells were illuminated with a 559-nm, 50-mW laser (WS-0559-050, NTT Electronics) or a 532-nm, 100-mW laser (Compass 315M-100) with an ND50 filter for TIRF; with a 559-nm, 50-mW laser (WS-0559-050, NTT Electronics) or a 532-nm, 100-mW laser (Compass 315M-100) with an ND50 filter for TIRF; with a 488-nm, 30% output power of 200-mW laser (Sapphire 488-200, Coherent) for GFP; or with a 637-nm, 140-mW laser (OBIS 637,
Coherent) for SiR and SF650 through the objective (PlanApo 60×, numerical aperture of 1.49; Nikon) by a dichroic mirror for TMR and GFP (FF493/574, Semrock) and for TMR and SiR (ET Cy3/Cy5, Chroma) or by a single-band filter set for SF650 (ET Cy5, Chroma). The emission light from TMR and GFP/SiR was split into two light paths by a two-channel imaging system (M202, Nikon) with a dichroic mirror [59004b (Chroma) for TMR and GFP or FF640-FDi01 (Semrock) for TMR and SiR] and simultaneously detected by two electron-multiplying charge-coupled device cameras (ImagEM, Hamamatsu) after passing through band-pass filters [ET525/50m and ET605/70m (Chroma) for TMR and GFP or FF640-FDi01 (Semrock) for TMR and SiR] and passively filtered by a single-band filter set for SF650 (ET Cy5, Chroma). The emission light from TMR and GFP/SiR was split into two light paths by a two-channel imaging system (M202, Nikon) with the following settings: exposure gain, 200; spot noise reduction, on. The cells were fixed to MetaMorph, Molecular Devices) with the following settings: exposure time, 30.5 ms for single-color or 33 ms for dual-color imaging; electron-multiplying gain, 200; spot noise reduction, on. The cells were fixed to evaluate the accuracies of the positions of TMR-labeled mGluR3, SiR-labeled Gα and GFP-labeled CLC, which was performed according to a previous method (46). The cells on a coverslip were treated with 4% paraformaldehyde/0.2% glutaraldehyde in PBS for 30 min at room temperature, and they were washed five times with HBSS before imaging.

**Analysis of single-molecule images**

The multiple TIFF files (16-bit) were processed by ImageJ software as follows. Background subtraction was performed with a rolling ball radius of 25 pixels, and two-frame averaging of the images was then performed with the Running_ZProjector plugin (Vale Lab home page; http://valelab.ucsf.edu/~ntstuurman/jplugins/). The dual-color images were aligned with the GridAligner plugin (VALE LAB) based on an affine transform algorithm. The two channels were calibrated with scattering images of gold particles (60 nm) recorded on the same day. To keep the single-molecule intensity constant across the images, the display range of the brightness and contrast was set at a constant range (minimum, 0; maximum, 1800), followed by image conversion to avf format (8-bit) without compression. SMT analysis was performed with G-Count software (G-Angstrom) based on a two-dimensional, Gaussian-fitting algorithm with the following parameters: region of interest size, 6 pixels; fluorescence limit, 12 arbitrary units; loop, seven times; minimum number of frames, 15. The calculation of the parameters from trajectories, the curve fittings, and the illustrations in the figures were obtained with Igor Pro 6.36 (WaveMetrics) as follows. The MSD within time nΔt of each trajectory was calculated by (22)

$$\text{MSD}(n\Delta t) = \frac{1}{N - 1 - n} \sum_{j=1}^{N-n} \left[ (x(j\Delta t + n\Delta t) - x(j\Delta t))^2 + (y(j\Delta t + n\Delta t) - y(j\Delta t))^2 \right]$$

(1)

where n is the length of frames, Δt is the frame rate (30.5 ms), and N is the total frame number of the trajectory. D Av was calculated on the basis of the two-dimensional diffusion equation

$$D_{Av}(n\Delta t) = \frac{1}{M} \sum_{j=1}^{M} \frac{\text{MSD}_j(n\Delta t)}{4n\Delta t}$$

(2)

where MSDj is the MSD of the jth trajectory and M is the total number of trajectories. D Av in the present study was calculated for n = 6 (nΔt = 183 ms). The EC50 and IC50 values of the ligand-dependent changes in D Av were calculated by Eqs. 3 and 4, respectively.

$$f(x) = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + \frac{x}{x_c}}$$

(3)

$$f(x) = \text{Top} + \frac{\text{Bottom} - \text{Top}}{1 + \frac{x}{x_c}}$$

(4)

The MSD-Δt plot was fitted as described previously (47)

$$\text{MSD}(\Delta t) = L^2 \left( 1 - \exp \left( -\frac{12D\Delta t}{L^2} \right) \right)$$

(5)

where L is the confinement length and D is the diffusion coefficient taking the limit of Δt to 0. The histogram of the displacement (r = √(MSD)) within Δt (30.5 ms) of the trajectories of each HMM diffusion state was fitted as described previously (48)

$$P(r) = \frac{r}{2D\Delta t} \exp \left( -\frac{r^2}{4D\Delta t} \right)$$

(6)

The histogram of the intensity distribution was fitted by the sum of the N Gaussian

$$P(x) = \sum_{n=1}^{N} A_n \exp \left( -\frac{(x-nI)^2}{2n\sigma^2} \right)$$

(7)

where n is the oligomer size, and I and σ are the mean and SD of a single TMR molecule, respectively. N was determined by using the Akaike information criterion. I and σ were estimated to be 530 and 210, respectively, from the measurement of TMR-labeled CD86. The percentage of each oligomer size, Percent(n), and mean oligomer size on each cell surface were calculated by Eqs. 8 and 9, respectively.

$$\text{Percent}(n) = \frac{100\sqrt{2\pi n\sigma A_n}}{\sum_{n=1}^{N} \sqrt{2\pi n\sigma A_n}}$$

(8)

Mean oligomer size = \frac{\sum_{n=1}^{N} n \text{ Percent}(n)}{100}$

(9)

Colocalization between TMR-labeled mGluR3 and SiR-labeled Gα protein or GFP-labeled CLC was defined as a distance of <100 nm between different proteins in the same frame that were in the same diffusion state, as estimated by VB-HMM analysis. It is difficult to distinguish between random colocalization and a true interaction based solely on the distance between two molecules within an image, and the analysis program without the criterion of the proteins being in the same diffusion state defined an interaction as a fast-moving Gα protein passing an immobile mGluR3 molecule. It is expected that two molecules are in the same diffusion state if they are truly coupled and moving together. In the VB-HMM analysis of mGluR3, Gα protein, and CLC, the single-frame displacement histograms were similarly divided into...
four diffusion states, whereas the fraction of each state was different depending on the protein (fig. S9). The position accuracies of TMR-labeled mGluR3, SiR-labeled G_{\alpha} and GFP-labeled CLC on the fixed cells were estimated to be 28, 24, and 31 nm, respectively, from 1 SD of the displacement distribution of the immobile particles. The error of the alignment between the two channels after image processing was estimated to be 18 from the difference in the positions of the same gold particles. Therefore, 100 nm corresponded to ~2 SDs of the total position accuracy. The time constants of colocalization were estimated from a curve fitting of the cumulative histogram (Figs. 4F and 5F) by the double-exponential Eq. 8.

\[ P(t) = A_1 \exp\left(-\frac{t}{\tau_1}\right) + A_2 \exp\left(-\frac{t}{\tau_2}\right) \]  

The fraction of two components was estimated from the ratio of \( A_1 \) to \( A_2 \).

**VB-HMM clustering analysis**

The VB-HMM analysis was performed with a LabVIEW-based homemade program developed according to previously reported algorithms (27,28). A trajectory of an mGluR3 molecule consists of a time series of step displacements. Each time series of the observed data is given as \( X = \{x_1, ..., x_N\} \), where \( N \) is the total number of frames. A corresponding time series of diffusion states is defined as \( Z = \{z_1, ..., z_N\} \). Here, \( z_n = \{z_{nk}, z_{nk}\} \), in which \( K \) is the total number of states, and \( z_{nk} = 1 \) when a molecule is in the \( k \)th state and \( n \)th frame, and 0 otherwise. To estimate the state series \( Z \) from the data \( X \), we applied the HMM where \( Z \) is assumed to obey the Markov process with transition matrix \( A \). The distributions of the initial state and transition probability are described as follows.

\[ p(z_1|\pi) = \prod_{i=1}^{K} \pi_i^{z_{1i}} \]  
\[ p(z_n|z_{n-1}, A) = \prod_{i=1}^{K} \prod_{j=1}^{K} A_{ij}^{z_{nj-1}z_{nj}} \]

where \( \pi_i = p(z_{1i} = 1) \) satisfies \( 0 \leq i \leq 1 \) and \( \sum_{i=1}^{K} \pi_i = 1 \), and \( A_{ij} \) is an element of \( A \) from the \( i \)th to \( j \)th state and satisfies \( 0 \leq A_{ij} \leq 1 \). The distribution of the emission probability, which represents the observation probability of the step displacement, is described with parameters \( \phi \) as follows.

\[ p(x_n|z_n, \phi) = \prod_{k=1}^{K} p(x_n|z_n, \phi)^{z_{nk}} \]

The probability is described with a two-dimensional diffusion equation

\[ p(x_n|z_n, D_k) = \frac{x_n^2}{2D_k\delta t} \exp\left(-\frac{x_n^2}{4D_k\delta t}\right) \]

where \( D_k \) is the diffusion constant of state \( k \), and \( \delta t \) is the frame rate (30.5 ms).

Thus, the joint probability distribution, \( p(X, Z | \theta) \), is

\[ p(X, Z | \theta) = p(z_1 | \pi) \prod_{n=2}^{K} p(z_n | z_{n-1}, A) \prod_{m=1}^{K} p(x_m | z_m, \phi) \]

where \( \theta = \{\pi, A, \phi\} \) represents the parameters of the observation probability. Molecular states \( Z \) and model parameters \( \theta \) were estimated by the VB method (49) to satisfy the maximum value of the logarithmic likelihood function of \( p(X) \). When the distribution of \( Z \) is specified by the model \( M \)

\[
\ln p(X) = \ln \sum_z p(X, Z | \theta) d\theta \\
= \sum_z [q(Z, \theta) \ln \frac{q(Z, \theta)}{p(Z, \theta | X, M)}] d\theta \\
= KL(q || p) + L_q
\]

where \( KL(q || p) \) is the Kullback-Leibler divergence between the distribution of model \( p \) and posterior function \( q \). Because \( KL(q || p) \) has fixed values for \( M \) and observable \( X \), \( L_q \) corresponds to the lower bound of \( \ln P(X) \). When \( q(Z, \theta) \) is assumed to be factorized as

\[ q(Z, \theta) = q(Z)q(\theta) = q(Z) \prod_{i=1}^{I} q(\theta_i) \]

To optimize the distribution functions \( q(Z) \) and \( q(\theta) \), the VB-EM (expectation-maximization) algorithm was applied (49). The VB-E step and VB-M step maximize \( L_q \) against \( q(Z) \) and \( q(\theta) \), respectively. The VB-E step corresponds to the calculation of

\[ \ln q(Z) = E_0[\ln p(X, Z, \theta | M)] + \text{const} \\
= \int q(\theta) \ln p(X, Z, \theta | M) d\theta + \text{const} \]

where \( E_0[...] \) represents the expectation with respect to \( \theta \). Thus

\[ q(Z) \propto \exp\left\{\int q(\theta) \ln p(X, Z, \theta | M) d\theta \right\} \]

By taking Eq. 15 into Eq. 20 and incorporating \( M \)

\[ q(Z) \propto \tilde{p}(z_1 | \pi, M) \times \prod_{n=2}^{K} \tilde{p}(z_n | z_{n-1}, A, M) \times \prod_{m=1}^{K} \tilde{p}(x_m | z_m, \phi, M) \]

On the basis of Eqs. 11 to 13, each term of Eq. 21 becomes

\[ \tilde{p}(z_1 | \pi, M) = \prod_{i=1}^{K} \exp({\ln \pi_i})^{z_{1i}} \]

\[ \tilde{p}(z_n | z_{n-1}, A, M) = \prod_{i=1}^{K} \prod_{j=1}^{K} \exp({\ln A_{ij}})^{z_{nj-1}z_{nj}} \]
where the overhead lines denote averages. \( q(Z) \) is optimized by the forward-backward algorithm using Eqs. 22 to 24. Similarly, the VB-M step corresponds to the calculation of

\[
\ln q(\theta_j) = E_Z[\ln p(X, Z, \theta|M)] + \text{const}
\]

\[
= \sum_Z q(Z) \prod_{i<j} q(\theta_i) \ln p(X, Z, \theta|M) d\theta_i + \text{const}
\]

Thus

\[
q(\theta_i) \propto \exp \left\{ \sum_Z q(Z) \prod_{i<j} q(\theta_i) \ln p(X, Z, \theta|M) d\theta_i \right\}
\]

\( q(\theta_i) \) can be factorized to separate terms for each parameter. By optimizing \( q(\theta_i) \), the expectations of parameters are obtained and used as updated values in the next VB-E step. The Dirichlet distribution was used for given prior functions of the initial state, transition probability distributions, and the calculated posterior functions. Thus, the log of expectation of the transition probability is

\[
\langle \ln p_{ij} \rangle = \psi(u_i^x + x_{ij}) - \psi \left( \sum_i u_i^x + 1 \right)
\]

where \( \psi(x) \) is the digamma function, \( \psi(x) = \frac{d}{dx} \ln \Gamma(x) = \frac{\Gamma'(x)}{\Gamma(x)} \) and \( u_i^x \) is the hyperparameter of the prior function and given as a flat probability distribution, \( u_i^x = 1 \). The log of expectation of the transition probability is

\[
\langle \ln A_{ij} \rangle = \psi(u_{ij}^D + \sum_{n=2}^{N} x_{n-1,j} x_{n,j}) - \psi \left( \sum_j \left( u_{ij}^D + \sum_{n=2}^{N} x_{n-1,j} x_{n,j} \right) \right)
\]

where \( u_{ij}^D \) is the hyperparameter and \( u_{ij}^D = 1 \). For the emission probability of a two-dimensional diffusion equation (Eq. 14), the prior function, including the diffusion coefficient \( (D_k) \), is given by a \( \gamma \) distribution, and the log expectation of parameter \( \tau_{D_k} \) (=1/2\( D_k \delta t \)) is

\[
\langle \ln \tau_{D_k} \rangle = \psi \left( a_D + \sum_{n=1}^{N} x_{n,j} \right) - \ln \left( b_D + 1 \right) / \left( 2 \sum_{n=1}^{N} x_{n,j} \right)^2
\]

where \( a_D \) and \( b_D \) are the hyperparameters and assigned values to maximize the lower bound \( L_q \) which was rewritten from Eq. 18 as

\[
L_q = E[\ln p(\pi)] + E[\ln p(A)] + E[\ln p(\psi)] - E[\ln q(\pi)] - E[\ln q(A)] - E[\ln q(\psi)] + \sum_{n=1}^{N} \ln c_n
\]

where \( c_n \) is the scaling factor calculated in the VB-E step. The iteration between the VB-E and VB-M step is performed until the lower bound converges. The VB-HMM analysis on the obtained data was carried out using the following procedure: First, we set the number of states \( (N) \) and divided the data into \( N \) groups by the K-means clustering method in which initial values were given by the K means++ method. Second, we calculated the initial parameters of the observation probability for each group. Third, we used the VB-E step to optimize \( q(Z) \) by Eqs. 22 to 24 with the forward-backward algorithm. Fourth, we used the VB-M step to update the parameters by Eqs. 27 to 29. Fifth, we calculated the lower bound, \( L_q \), by using Eq. 30 and judging its convergence, except for the first \( L_q \) by determining whether the difference from the previous \( L_q \) was <0.001%. Sixth, if \( L_q \) was not converged, we repeated the next iteration step by repeating steps three to five. Seventh, we optimized the state sequence by choosing a state with the highest probability at every frame. For the calculation in the fourth step, we assigned \( u_i^x = 1 \) and different fixed values for \( a_D \) and \( b_D \) that gave maximum lower bounds for the observed trajectory data.

Heterologous expression and membrane preparation for in vitro biochemical assay

Heterologous expression of mGluR3 in HEK 293 cells for in vitro biochemical assay was performed according to previously reported methods (20). HEK 293 cells growing to ~40% confluency in DMEM/F12 medium supplemented with 10% FBS were transfected with plasmid DNA (10 µg/100-mm dish) encoding mGluR3 or the pCAG vector (mock) by the calcium phosphate method. The cells were collected 48 hours after transfection by centrifugation, and the pellet was washed with 1 ml of PBS (pH 7.4). The cell pellet (in a 1.5-ml tube) was homogenized with a pellet mixer in 50% sucrose in buffer A [50 mM Hepes (pH 6.5), 140 mM NaCl] before centrifugation. The supernatant containing the plasma membrane was diluted in two volumes of buffer A and recentrifuged. The membrane pellet was then washed with buffer A and stored at ~80°C.

Western blotting

The mGluR3-containing membrane pellet was suspended in sample buffer [62.5 mM tris–HCl (pH 6.8), 4% SDS, 10% glycerol] with or without 2.5% β-mercaptoethanol (ME). After being resolved by 5.5% SDS–polyacrylamide gel electrophoresis (PAGE), the electrophoresed proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and incubated with the Rho1D4 antibody (primary antibody) and the horseradish peroxidase (HRP)–linked anti-mouse immunoglobulin G (secondary antibody; 7076, Cell Signaling Technology). Immunoreactive proteins were detected with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) using ImageQuant LAS 500 (GE Healthcare). To estimate the efficiency of RNAi-mediated knockdown of CLC, we transfected the cells with the siRNAs as described earlier for the single-molecule imaging experiments. After 2 days, the cells were harvested and homogenized with a pellet mixer. The cell lysate was boiled for 20 min in sample buffer containing 2.5% ME. After subjecting the lysate to 15% SDS-PAGE and transfer to a PVDF membrane, CLC and β-actin were detected with anti-CLC and anti-β-actin monoclonal antibodies, respectively. The HRP-linked secondary antibody reaction and detection of immunoreactive proteins were performed as described earlier. The density of bands was quantified by ImageJ software.

[^3H]Ligand-binding assay of mGluR3

Cell membranes containing mGluR3 were resuspended in HBSS [with 15 mM Hepes (pH 7.1), without NaHCO₃ (Sigma-Aldrich)], which are the same buffer conditions as for the single-molecule imaging experiments. [^3H]LY341495 binding to membranes was measured at...
room temperature. The membranes (5 μg of total protein) were incubated with 0 to 1 μM [3H]LY341495 in HBSS for 30 min (final assay volume, 20 μl). After incubation, bound and free radioligands were separated by filtration through a nitrocellulose membrane (0.45-μm HATF, Millipore) using a dot-blotter (FLE396AA, ADVANTEC). The nitrocellulose membrane was washed twice with HBSS (200 μl) and dried for 1 h. The pieces of the nitrocellulose membrane were put in scintillation cocktail (Ultima Gold, PerkinElmer), and the bound [3H]LY341495 was quantified with a liquid scintillation counter (LS6500, Beckman Coulter). Nonspecific binding was measured using 20 μl of 200 nM GDP. After incubation for 30 min at 37°C, the reaction was terminated by adding 200 μl of stop solution [20 mM tris-Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl2, 500 nM GTPγS (cold), and 500 nM GDP]. After incubation for 30 s, the reaction was terminated by adding 200 μl of stop solution [20 mM tris-Cl (pH 7.4), 100 mM NaCl, 25 mM MgCl2, 500 nM GTPγS (cold), and 500 nM GDP] and immediately filtering the sample through a nitrocellulose membrane (0.45-μm HATF, Millipore) to trap [35S]GTPγS bound to G proteins. The nitrocellulose membrane was washed three times with 200 μl of buffer C [20 mM tris-Cl (pH 7.4), 100 mM NaCl, and 25 mM MgCl2] and dried for 1 h. The pieces of the nitrocellulose membrane were put in scintillation cocktail (Ultima Gold, PerkinElmer), and the bound [3H]GTPγS was quantified with a liquid scintillation counter (LS6500, Beckman Coulter). Nonspecific binding was measured using the mock-transfected HEK 293 cell membrane.

Saturation binding assay of TMR, SF650, and SiR

HEK 293 cells cultured to ~90% confluence on a 100-mm dish were detached using the same protocol used to passage the cells and suspended in 4 ml of DMEM/F12 with 10% FBS. After 15 min of incubation at room temperature, the transfection mixture [plasmid DNA of HaloTag-fused mGluR3, SNAP-tag fusion Gαs, or pcDNA3.1 vector (2 μg), P3000 reagent (4 μl), Lipofectamine 3000 reagent (5 μl), and Opti-MEM (240 μl; Gibco)] was added to the cell suspension (0.5 ml). After 2 min of incubation at room temperature, the Lipofectamine-treated cells were diluted with 6 ml of DMEM/F12 with 10% FBS. The cell suspension (100 μl) was added to each well of a black, collagen I-coated, 96-well plate (Nunc, Thermo Fisher Scientific). After overnight incubation at 37°C in 5% CO2, the medium in each well was exchanged for 0 to 3 μM TMR, SF650, or SiR ligand solution in DMEM/F12 without phenol red (50 μl). After 15 min of incubation at 37°C in 5% CO2, the cells were washed three times with 100 μl of DMEM/F12 without phenol red, and the medium was finally replaced with 100 μl of 0.001% BSA/HBSS before quantification. Saturation binding of the HaloTag ligand was detected by a microplate reader (FlexStation 3, Molecular Devices) with the following parameters: mode, fluorescence; excitation/cutoff/emission, 530/570/580 nm for TMR and 640/665/675 nm for SF650 and SiR; photomultiplier gain, automatic; flashes per read, 6; read from bottom. The background fluorescence intensity was estimated from the intensity of cells without HaloTag ligand treatment. Nonspecific binding was determined by the fluorescence intensity of mock-transfected cells. Specific binding was calculated as the difference between the total binding to cells expressing HaloTag-fused mGluR3 and the nonspecific binding. The data were fitted with the Hill equation:

\[ f(x) = \frac{\text{Bottom} - \text{Bottom}_n}{(1 + \frac{K_{n}}{C_0})^{n}} \]

where n is the Hill coefficient.

**SUPPLEMENTARY MATERIALS**

www.sciencesignaling.org/cgi/content/full/11/548/eaao1917/DC1

Fig. S1. Evaluation of the effect of the HaloTag fusion to mGluR3.
Fig. S2. Comparison of HaloTag and SNAP-tag ligands by single-molecule imaging.
Fig. S3. Example of the VB-HMM analysis of mGluR3 trajectories.
Fig. S4. Dose-dependent change in the time constants of the state transition.
Fig. S5. Dose-dependent change in the diffusion coefficient of each diffusion state.
Fig. S6. Correlation between mean oligomer size and receptor density under various ligand conditions.
Fig. S7. Correlation between Dms and receptor density under various ligand conditions.
Fig. S8. MSD-σt plots of the trajectories of GPCRs with or without agonist.
Fig. S9. Histograms showing displacement during 33 ms of the trajectories for mGluR3, Go protein, and CLC molecules, categorized into four diffusion states, using VB-HMM analysis.

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