The bradykinin receptor is a G protein-coupled receptor (GPCR) that is coupled to the G\textsubscript{\alpha}\text{q} family of heterotrimeric G proteins. In general, a GPCR can exert intracellular signals either by transiently associating with multiple diffusing G protein subunits or by activating a G protein that is stably bound to the receptor, thus generating a signal that is limited by the stoichiometry of the complex. Here we have distinguished between these models by monitoring the association of type 2 bradykinin receptor (B2R) and the G\text{\alpha}\text{q}/G\beta\gamma heterotrimer in living human embryonic kidney 293 cells expressing fluorescent-tagged proteins. Stable B2R-G\text{\alpha}\text{q}-G\beta\gamma complexes are observed in resting cells by fluorescence resonance energy transfer from either G\text{\alpha}\text{q}-eCFP or eCFP-G\beta\gamma to B2R-eYFP. Stimulating the cells with bradykinin causes detachment of B2R from the G protein subunits as the receptor internalizes into early endosomes, with a corresponding elimination of B2R-G protein fluorescence resonance energy transfer because G\text{\alpha}\text{q} and its associated G\beta\gamma remain on the plasma membrane. Single point and scanning fluorescence correlation spectroscopy measurements show that a portion of B2R molecules diffuses with a mobility corresponding to dimers or small oligomers, whereas a second fraction diffuses in higher order molecular assemblies. Our studies support a model in which receptors are pre-coupled with their corresponding G proteins in the basal state of cells thereby limiting the response to an external signal to a defined stoichiometry that allows for a rapid and directed cellular response.

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The organization of GPCRs in their natural cellular environment is unknown. There is increasing evidence that GPCRs form homo- and heterodimers and oligomers, and this complex formation modifies their response to agonists and their interaction with downstream signaling molecules (for review see Ref. 3). However, the relative population of GPCRs in the monomeric, dimeric, and higher oligomeric states is unknown, and it is also not known whether GPCR dimers are complexed with their corresponding G proteins in larger molecular assemblies in living cells. In this study, we have distinguished between the two signaling models described above using the bradykinin-B2R-G\text{\alpha}\text{q}/G\beta\gamma-PLC\beta signaling system.

Bradykinin (BK) is a key mediator of the inflammation response and binds strongly to its two corresponding G protein-coupled receptors, B1R and B2R. B2R is only expressed in certain inflammatory and pathologic states, unlike B2R, which is constitutively expressed (4). Stimulation of B2R by BK causes activation of the G\text{\alpha}\text{q} family of heterotrimeric G proteins (5). Activated G\text{\alpha}\text{q} stimulates the activity of phosphatidylinositol-specific phospholipase C\beta (PLC\beta). Inositol-specific PLCs catalyze the hydrolysis of a minor component in lipid membranes, phosphatidylinositol 4,5-bisphosphate, to produce two second messengers that lead to an increase in intracellular calcium and activation of protein kinase C (for review see Ref. 6). Thus, addition of BK to cells expressing B2R results in a corresponding increase in intracellular Ca\textsuperscript{2+}, leading to a host of mitogenic and proliferative changes.

Upon the binding of agonist BK, B2R is desensitized by phos-
phorylation on serine and threonine residues on its C terminus (7, 8). It is then sequestered into vesicles and internalized (9–12). If Goq and GBγ subunits are bound to receptor in the basal state, then their fate during and after receptor desensitization is uncertain. It has been found that Goq and GBγ subunits coupled to βγ-adenoreceptor receptors dissociate from the membrane as the agonist-bound receptors internalize (13). Whether B2R internalization affects the localization of Goq and GBγ is unknown.

In this study, we have assessed the organization of Goq/GBγ and B2R by FRET using eCFP/eYFP-tagged proteins (14). We measured the mobility of the GFP-tagged receptor using FCS (15–18) and scanning FCS techniques (19) in human embryonic kidney 293 (HEK293) cells, and we related the mobility to the size of the receptor-G protein complexes. We also find that B2R receptors are pre-coupled with G proteins in the basal state supporting a stoichiometric model of signaling. We find that a significant population of B2R-G protein complexes exists in higher order structures on the membrane surface thus allowing simultaneous stimulation of a number of G protein subunits.

**EXPERIMENTAL PROCEDURES**

Materials—G protein constructs eCFP-GBγ, GFP-GBγ, HAγ2, and Goq-eCFP were provided by Dr. Catherine Berlot (Geisinger Clinic, Danville, PA). Human B2R and B2R-GFP constructs were provided by Dr. Frederick Leeb-Lundberg (University of Texas, San Antonio, TX). Fluorescent tags are inserted between residues 124 and 125 (opposite the membrane-binding site) of murine Goq and on the N terminus of human GBγ. The constructs used in this study, when expressed in cells, were found to activate PLC. eYFP-PLCδ1, used as a negative control in the FRET studies, was provided by Dr. Mario Rebecchi (Stony Brook University), and eCFP–(GS)5–eYFP, used as a positive FRET control, was provided by Dr. Jeffrey Pessin (Stony Brook University). EEA1 antibody (early endosomal marker) was a gift from Dr. Deborah Brown (Stony Brook University). EEA1 antibody detects all members of the G protein subfamilies and annexin V (endosomal marker) was a gift from Dr. Deborah Brown (Stony Brook University). EEA1 antibody detects all members of the G protein subfamilies.

Construction of B2R-eYFP Plasmid—B2R-GFP plasmid was modified to obtain B2R-eYFP. B2R is flanked by enzyme sites at the C terminus of B2R and is flanked by PacI enzyme site at its C terminus. GFP from B2R-GFP was digested out with MluI and was separated from its vector and then ligated to the B2R fragment with vector to obtain B2R-eYFP plasmid.

**Cell Culture and Transfection of HEK293 Cells**—HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO2 incubator. Cells were transfected by calcium phosphate coprecipitation in which 5–10 μg of plasmid was mixed with 120 mM CaCl2 and HBS buffer (21 mM Heps, 123 mM NaCl, 5 mM KCl, and 0.9 mM Na2HPO4, pH 7.1), incubated on ice for 10 min, and added to cells maintained in 60-mm dishes. The medium of the cells was replaced after overnight incubation.

**Determination of Levels of Endogenous GBγ and Goq and Overexpressed GFP-B2R in HEK293 Cells**—Western blot band densities corresponding to known amounts of purified GBγ or Goq (standard) and those corresponding to GBγ or Goq on the natural membranes were measured and used to estimate the amount of GBγ and Goq on the natural membranes. We note that samples were measured in the linear range of the standard curves made using purified proteins. An average of 90 ng of GBγ and 30 ng of Goq was obtained per 10 μg of total membrane proteins loaded in the lanes. Therefore, the amounts of GBγ and Goq relative to the total membrane protein present on the natural membranes were 0.9 and 0.3%, respectively.

Based on these calculations, the amounts of endogenous GBγ and Goq on the natural membranes, which were titrated into the cuvette in the binding experiments, were determined. The total amount of membrane proteins present after titration was 0.6 μg, with a total endogenous GBγ of 5.4 ng and endogenous Goq of 1.8 ng, which corresponds to 3 nM endogenous GBγ and 1 nM endogenous Goq. The amount of endogenous GBγ and Goq in a single cell was estimated by dividing the amount of proteins by the total number of cells from which the membranes were prepared.

To estimate the amount of overexpressed GFP-B2R, we compared the fluorescence intensities from HEK293 cell membranes to known concentrations of PH(PLCδ1)-GFP. The overexpressed level of B2R-GFP is ~37 pm.

**Western Blotting**—Proteins in HEK293 cell fractions were separated through gel electrophoresis and transferred from an SDS gel onto a nitrocellulose membrane. After blocking the membrane with 1% BSA, the membrane was incubated with primary antibody. Primary antibody for Goq or GBγ was used to immunoblot these proteins. This was followed by three washes and incubation with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase. The bands were then detected by adding color-developing reagents.

**Immunofluorescence to Label Early Endosomes**—HEK293 cells overexpressing B2R-GFP were washed twice with PBS followed by incubation with 1 μM BK. Cells were fixed with 3% paraformaldehyde, incubated for 10 min, and washed three times with Modified Shields Media/PIPES buffer (18 mM MgSO4, 5 mM CaCl2, 40 mM KCl, 24 mM NaCl, 5 mM PIPES, pH 6.8, 0.5% Triton X–100, and 0.5% Nonidet P-40) for 7 min each. Cells were blocked in PBS containing 5% goat serum, 1% BSA, and 50 mM glycine for 15 min. EEA1 monoclonal mouse antibody diluted to 1:100 in PBS + 1% BSA was added followed by incubation at 37°C for 45 min. The antibody was removed, and the cells were washed three times with PBS + 1% BSA for 10 min each. This was followed by the addition of anti-mouse Texas Red secondary antibody, which was diluted to 1:200 in PBS + 1% BSA, and subsequent incubation at 37°C for 45 min.
After washing the cells three times each for 10 min with PBS, they were viewed in PBS under a fluorescent microscope.

**Measurement of Cellular Calcium**—Cellular calcium levels were determined with the fluorescent calcium indicator dye Fura-2-AM (Molecular Probes, Inc.) in an ISS spectrophotometer. HEK293 cell monolayers were washed two times with Hanks’ balanced salt solution (HBSS) (118 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, 15 mM Hepes, 1% BSA, pH 7.4) and were detached by a buffer stream. Suspended cells were counted, labeled with 1 μM Fura-2-AM for 45 min at 37 °C in HBSS with rotation, and washed twice to remove extracellular dye. Cell density was adjusted to 1 × 10⁶ cells/ml in HBSS, and the suspension was placed in a cuvette, and the ratio of fluorescence intensities at excitation wavelengths at 340 and 380 nm was taken by monitoring the emission at 510 nm. The intensity ratios were monitored over a period of 10 min during which the cells were stimulated and lysed with 10% Triton followed by addition of 400 nM EDTA. Each event was separated by a time interval of 2 min. The ratio was converted into calcium concentration with the relation (20): [Ca²⁺] free (nm) = \((R - R_{\text{min}})/(R_{\text{max}} - R) \times (F_{\text{max}}/F_{\text{min}}) \times K_d (nm)\), where \(K_d \sim \text{225 nM; } F_{\text{max}}\) is the fluorescence in EDTA buffer, and \(F_{\text{min}}\) is the fluorescence in the presence of detergent-excess calcium. \(R\) is the measured ratio, and \(R_{\text{min}}\) and \(R_{\text{max}}\) are the ratios corresponding to the EDTA and detergent-excess calcium conditions, respectively.

**Membrane Binding Studies**—Membranes from wild type HEK293 cells or ones overexpressing B₂R were prepared by washing harvested cells twice with PBS and centrifuging at 3000 rpm for 5 min. A mixture of phosphate-buffered saline, phenylmethylsulfonyl fluoride, aprotinin, and protease inhibitor mixture for mammalian cells was added, and the cells were homogenized and centrifuged at 3000 rpm for 5 min in 4 °C. The supernatant was then spun at 50,000 × g for 20 min in 4 °C. The suspension was then spun at 3000 rpm for 5 min in 4 °C. The supernatant was then spun at 3000 rpm for 5 min in 4 °C. The suspension was placed in a cuvette, and the ratio of fluorescence intensities at excitation wavelengths at 340 and 380 nm was taken by monitoring the emission at 510 nm. The intensity ratios were monitored over a period of 10 min during which the cells were stimulated and lysed with 10% Triton followed by addition of 400 nM EDTA. Each event was separated by a time interval of 2 min. The ratio was converted into calcium concentration with the relation (20): [Ca²⁺] free (nm) = \((R - R_{\text{min}})/(R_{\text{max}} - R) \times (F_{\text{max}}/F_{\text{min}}) \times K_d (nm)\), where \(K_d \sim \text{225 nM; } F_{\text{max}}\) is the fluorescence in EDTA buffer, and \(F_{\text{min}}\) is the fluorescence in the presence of detergent-excess calcium. \(R\) is the measured ratio, and \(R_{\text{min}}\) and \(R_{\text{max}}\) are the ratios corresponding to the EDTA and detergent-excess calcium conditions, respectively.

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The binding of Gₐq or Gβγ subunits to natural membranes was carried out by labeling with the thiol-reactive probe CPM as described previously (21). Binding was measured on the proteins immediately after dialysis that removed storage detergent. Natural membrane fractions from HEK293 cells were added to CPM-labeled Gβγ or Gₐq subunits, and changes in CPM emission spectra were monitored exciting at 384 nm and scanning the emission spectrum from 420 to 540 nm. After correcting for background scatter and dilution, the normalized changes in emission energies were plotted and fit to obtain an apparent partition coefficient, \(K_p\).

FRET between RITC-Gβγ and B₂R-GFP was carried out by labeling Gβγ subunits with N-terminal reactive probe RITC (X rhodamine) by adding a 2-fold excess of RITC after dialyzing Gβγ in 0.1 mM sodium bicarbonate and β-mercaptoethanol buffer, pH 9.0. After incubation on ice for 30 min, RITC-Gβγ was dialyzed in buffer containing 150 mM NaCl, 20 mM Hepes, pH 7.2, and 1 mM dithiothreitol to remove the excess probe. RITC-labeled Gβγ was titrated into cuvette containing natural membranes from HEK293 cells expressing B₂R-GFP. Titration of Gβγ-RITC into a cuvette containing control buffer was recorded for background subtraction. B₂R-GFP was excited at 470 nm, and emission spectra were scanned from 500 to 660 nm to obtain both donor and acceptor peaks for each trial. Decrease in donor and the corresponding increase in acceptor emissions were recorded and analyzed to obtain an apparent dissociation constant for Gβγ and B₂R.

**Fluorescence Imaging and FRET in Cells**—Images of cells overexpressing fluorescent-tagged B₂R, Gβγ, and/or Gₐq were taken on Zeiss Axiovert 200M. HEK293 cells were viewed under the microscope in L-15 medium (without phenol red) with 0.1% BSA and 0.1% glucose. Cells were monitored for 48 h after stimulation to ensure that stimulation did not lead to the formation of any irregular characteristics. Imaging in two channels was done for immunofluorescence and colocalization experiments, and three-channel imaging was done for FRET measurements. The mean intensity of a region of interest was calculated using a plugin for the ImageJ software. Three-dimensional plots were constructed by taking the average fluorescence intensities along concentric circles at increasing distances from the center of a cell to the membrane and plotting the intensities against these distances as a function of time. Average intensities were calculated for each frame acquired over time and normalized to the highest value in each frame to correct for photobleaching (see below).

The percentage colocalization of B₂R-eYFP and eCFP-Gₐq or eCFP-Gβγ was analyzed using ImageJ software. Briefly, the images in grayscale from both eYFP and eCFP channels were corrected for background and converted to black and white. This was done by converting the pixel values in each image to either 0 (all pixels below a threshold value) or 255 (all pixels above the threshold value). The black and white images from both channels were then added to obtain a colocalized image. Two points were considered colocalized if their respective intensities were strictly higher than the threshold of their channels. The mean intensity of the resulting colocalized image was divided by the average of the intensities from the black and white images of the two channels to obtain a percentage of colocalization of the two proteins. Percentage of colocalization calculated with a plug-in available with the software provided us with similar values.

Energy transfer between eCFP-Gβγ-B₂R-eYFP and eCFP-Gₐq-B₂R-eYFP pairs was measured by taking images of cells coexpressing both proteins under three filters, CFP, YFP, and FRET (CFP, 31044v2, exciter D436/20x, dichroic 460DCLP, emitter D480/40m; YFP, 41029, exciter HQ500/20x, dichroic QS515LP, emitter HQ520LP; and FRET, 31052, exciter D436/20x, dichroic 460DCLP, emitter D535/30m). The FRET filter excites at the eCFP excitation wavelength and transmits light at the eYFP emission wavelength. FRET data were analyzed by taking the intensities of images acquired through the FRET (IᶠRET), CFP (IᶠCFP), and YFP (IᶠYFP) filters and first correcting for background. The resulting image intensity from each channel was then corrected for photobleaching that had occurred through the channel. This was done by dividing the image intensity by \(e^{-kt}\), where \(k\) is the rate of decay. Rate of decay for
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each filter was obtained by fitting intensity values from photobleached cells expressing either eCFP or eYFP to an exponential rise to maximum function in Sigmaplot. After correcting for photobleaching, a normalized FRET value was calculated with the following equation (see Ref. 22): $N_{\text{FRET}} = I_{\text{FRET}} - (I_{\text{CFP}} \times \alpha) - (I_{\text{YFP}} \times \beta) / ((I_{\text{CFP}} \times I_{\text{YFP}})^{1/2}$, where $\alpha$ and $\beta$ are CFP and YFP bleed through emissions through the FRET filter, respectively. Averaging over 12 cells on our system, the bleed through values for CFP and YFP channels are 39 and 28%, respectively, using the background-corrected intensities calculated using ImageJ software from the National Institutes of Health. A plug-in is created in ImageJ to correct for background and to calculate normalized FRET after bleed through corrections were used to obtain images showing pixel intensities that correspond to normalized FRET values.

Fluorescence Correlation Spectroscopy Measurements—FCS measurements were carried out using both 1-photon and 2-photon excitation. 1-Photon FCS experiments (only single point) were performed with an Axiovert 200 microscope with a ConfoCor2 unit equipped with a 40× (N.A. 1.2) water immersion objective (Apochromat) and a continuous argon ion laser (Carl Zeiss, Jena, Germany). Excitation of the fluorophore (GFP) was at 488 nm with an incident laser power of 20 microwatts, and the fluorescence was recorded using an avalanche photodiode through a long pass emission filter (LP505). The time trace of the fluorescence was analyzed by a digital temporal correlator to compute the autocorrelation function $G(\tau)$. The ConfoCor2 software controlled the experimental setup and produced the autocorrelation function. The focal volume for the FCS experiment was determined by calibrating the instrument with a 2 nm Alexa488 solution in which the diffusion constant is $D_{\text{Alexa488}} = 2.2 \times 10^{-6}$ cm²/s. All measurements were done at room temperature, ∼25 °C. Autocorrelation functions, $G(\tau)s$, were analyzed using the fitting routine for two-dimensional diffusion provided by Zeiss with their ConfoCor2 software. We used the following equation: $G(\tau) = \sum a_i (1 + \tau/\tau_i)^{-1}$, where $\tau_i$ is the residence time of a molecule in FCS observation volume, and $a_i$ is the relative contribution of the $i$th species in the autocorrelation. Diffusion coefficient, $D$, is calculated from $\tau_{\text{DF}}$ of a molecule using Einstein relation for diffusion: $r^2 = 4D \times \tau_{\text{DF}}$, where $r$ is the radius of the observation volume (for details see Refs. 15, 17, 23).

Single point FCS and scanning FCS measurements using 2-photon excitation were performed at the Laboratory of Fluorescence Dynamics (University of Illinois, Urbana-Champaign) headed by Dr. Enrico Gratton (University of California, Irvine). FCS measurements were performed at ∼25 °C as described (19, 24, 25) using excitation at 905 nm. A mode-locked titanium:sapphire laser with 80-MHz, 100-fs pulse width (Tsunami; Spectra-Physics, Mountain View, CA) was used as the excitation light source. The laser was guided into the microscope by $x$-$y$ galvano-scanner mirrors (model 6350; Cambridge Technology, Watertown, MA) to achieve beam scanning in both $x$ and $y$ directions. A voltage generated by a computer card moved the scanner mirrors. The movement of the $x$ scanner mirror was independent from the $y$ scanner mirror movement. For scanning FCS experiments with a circular laser beam orbit, the $x$ and $y$ scanner mirrors were driven by two identical sine waves with 90° phase shift. The radius and frequency of the circular scan were controlled by the amplitude and frequency of the sine waves. A photomultiplier tube (Hamamatsu R7400P, Japan) was used for light detection in the photon counting mode. A BG39 optical filter (Chroma Technologies, Brattleboro, VT) was placed before the photomultiplier. A Zeiss 40× (1.2 N.A.) water-immersion objective lens was used for the measurement. All data acquisition and analysis were done using SIMFCS and GLOBALS software developed in Laboratory of Fluorescence Dynamics (University of Illinois, Urbana-Champaign) by Prof. Gratton.

For single point FCS, the sampling rate was 64 kHz, and the total measurement lasted less than 5 min. For scanning FCS the orbital scanning frequency was 1 kHz with a sampling rate (pixel rate) of 64 kHz and total measurement times of 512 s. The laser power at the sample was 10 milliwatts. The average fluorescence intensity of the sample remained constant, indicating that the extent of photobleaching was negligible, if any. The waist $\omega_0$ of the excitation beam was calibrated each day by measuring an autocorrelation curve of a 10 nm fluorescein solution in 0.01M NaOH. The beam waist was derived from a fit to this curve by assuming a fluorescein diffusion coefficient of $3 \times 10^{-6}$ cm²/s; typically $\omega_0$ values of 0.3 μm-0.5 μm were obtained.

For single point FCS data, the experimental autocorrelation function was fitted using a three-dimensional Gaussian-Lorentzian beam profile (26, 27) and one- or two-dimensional diffusion equations (see above). The concentration (C) of the fluorescent species was calculated from the $G(0)$ value obtained from the autocorrelation fits according to the following equation: $C = \gamma/G(0)N_A V_{PSF}$, where $N_A$ is Avogadro’s number, and $V_{PSF}$ is the volume of the point spread function (∼1 femtoliter). The factor $\gamma$ accounts for the illumination of the excitation volume and equals 0.072 for the 2-photon excitation profile.

For scanning FCS measurement, the center of the circular scanning path was directly selected from the fluorescence image. The data acquisition frequency was 64 kHz, and the scanning frequency was 1 kHz, yielding 64 data points in each scanning cycle. The data were plotted on an intensity carpet where the $x$ axis is the position along the scan and the $y$ axis is time (19). The scanning diameter was ∼12 μm for the reported measurements shown in Figs. 8 and 9. Each column on the $x$ axis (total 64 for one experiment) was temporally correlated and fitted by using a procedure similar to that for the single point FCS data and is described in the following equation: $G(\tau) = G_{01}(1 + \tau/\tau_1)^{-1} + G_{02}(1 + \tau/\tau_2)^{-1} - B$, where $\tau_i$ represents the residence time of the molecules in the excitation volume. Each component contributes with amplitude $G_{0i}$ subtracted by the background $B$. The diffusion coefficients, $D$, were calculated from $\tau_i$ according to the equation $\tau_i = \omega_0^2/8D$ for 2-photon excitation.

RESULTS

Expression of Functional B2R, Gaq, and GBγ in Untransfected and Transiently Transfected HEK293 Cells—Because the tendency for proteins to associate depends on their concentrations, we determined whether overexpression of the proteins would promote complex formation. The cellular concentrations of GBγ and Gaq were estimated using Western blot analysis of
total cell lysate by comparing the band intensities to standard curves derived from different concentrations of purified proteins. An average of 90 ng of Gβγ and 30 ng of Goq per 10 μg of total membrane proteins loaded in the lanes was obtained. The amounts of Gβγ and Goq relative to the total membrane protein present on the natural membranes are 0.9 and 0.3%, respectively. The total amount of membrane proteins present after titration was 0.6 μg, with a total endogenous Gβγ of 5.4 ng and endogenous Goq of 1.8 ng, which corresponds to 3 nM endogenous Gβγ and 1 nM endogenous Goq. The amount of endogenous Gβγ and Goq in a single cell was estimated by dividing the amount of protein by the total number of cells from which the membranes were prepared and were found to be ~6 and 2 pm, respectively. The 3-fold higher amount of Gβγ as compared with Goq is reasonable because Gβγ also forms complexes with other types of Go subunits.

The expression of B2R is limited to certain cell types, and we first determined whether HEK293 cells contain this receptor. We could not detect the receptor by Western blot analysis, so we used a functional assay in which we determined whether the addition of bradykinin increases intracellular Ca2+, which would be the case if B2R was present. We treated the cells with 1 μM bradykinin and measured changes in intracellular Ca2+...
using the fluorescent calcium indicator Fura 2 AM (Fig. 1). In untransfected cells, stimulation with bradykinin resulted in a small increase in the level of intracellular Ca\(^{2+}\) but not above significance, suggesting that HEK293 cells contain little, if any, endogenous B\(_R\). In cells transfected with B\(_R\), the addition of bradykinin resulted in a significant increase in intracellular Ca\(^{2+}\). The overexpressed level of B\(_R\)-GFP in HEK293 cells is estimated to be \(\sim 37\) pm. To estimate the amount of overexpressed B\(_R\)-GFP, we compared the fluorescence intensities from HEK293 cell membranes to known concentrations of PH(PLC\(_{6}\))–GFP.

HEK293 cells are known to express acetylcholine receptors leading to large increases in Ca\(^{2+}\) levels. To better assess the increase in Ca\(^{2+}\) with bradykinin, we stimulated both wild type and B\(_R\)-GFP-transfected cells with 1 \(\mu\)M acetylcholine. Although wild type cells displayed a large release of intracellular Ca\(^{2+}\), this level was significantly decreased in B\(_R\)-GFP-transfected cells. This result suggests that overexpression of B\(_R\) receptors may sequester G proteins from other G protein-coupled receptors, such as acetylcholine receptors. In any case, these studies show that the B\(_R\) receptors in transfected HEK293 cells couple to G protein subunits in a functional manner.

**Cellular Localization and Movement of B\(_R\)**—We characterized the cellular distribution of B\(_R\)-GFP in transfected HEK293 cells by collecting images in slices having a thickness of 0.4 \(\mu\)M. These studies verified that the receptors are completely localized on the plasma membrane in the basal state and did not mis-localize because of overexpression or the attachment of the GFP tag (e.g. Fig. 2A). Stimulation of the cells with 1 \(\mu\)M bradykinin resulted in the internalization of the majority B\(_R\)-GFP within 30 s (Fig. 2B). Interestingly, the B\(_R\)-GFP containing vesicles did not penetrate deeply into the cytoplasm but stayed near the plasma membrane. Analysis of the \(z\)-stack images over time show that the vesicles move a maximum distance of \(\sim 2\) \(\mu\)M from the membrane, which is \(\sim 1/5\) of the total cellular distance based on estimation of the average cell length (Fig. 2C).

GPCRs have been shown to internalize through early endosomes. To verify that the internalization of B\(_R\)-GFP was endosomal, we carried out immunofluorescence studies with an antibody to the early endosome protein, EEA1, in HEK293 cells transfected with B\(_R\)-GFP. Cells were stimulated, fixed, and labeled with a fluorescence-tagged EEA1 antibody. The degree of colocalization was found to be 60 ± 3% (\(n = 4\)) indicating that B\(_R\) transits through early endosomes during internalization.

**Interaction of B\(_R\) and G Protein Subunits in HEK293 Cells**—We carried out a series of studies to determine whether the receptor is pre-complexed with G proteins in the basal state of cells. Initially, we tested whether G\(\beta\)\(\gamma\) subunits could interact directly with B\(_R\) because this would allow us to better interpret the cellular FRET experiments described below. In these experiments, we measured the degree of FRET between purified rhodamine-labeled G\(\beta\)\(\gamma\) and B\(_R\)-GFP on HEK293 membrane preparations by *in vitro* FRET measurements using membranes prepared from HEK293 cells as background. The FRET results in Fig. 3 show that these two proteins directly associate.

This observation corresponds well with the idea that G\(\beta\)\(\gamma\) subunits play a direct role in receptor signaling (28, 29).

To determine whether B\(_R\) complexes with G proteins in cells, we carried out a series of fluorescence microscopy studies. We first measured the amount of colocalization of eCFP-G\(\alpha\)_q and B\(_R\)-eYFP and eCFP-G\(\beta\)\(\gamma\) and B\(_R\)-eYFP in transfected HEK293 cells (see *Experimental Procedures*). These studies showed that 67 ± 2% (\(n = 8\)) of eCFP-G\(\alpha\)_q and B\(_R\)-eYFP and 63 ± 1% (\(n = 6\)) eCFP-G\(\beta\)\(\gamma\) and B\(_R\)-eYFP are colocalized (Fig. 4A) suggesting B\(_R\)-G\(\alpha\)_q–G\(\beta\)\(\gamma\) complexes. Stimulation of the cells with bradykinin resulted in internalization of B\(_R\) without a concomitant change in either G\(\alpha\)_q or G\(\beta\)\(\gamma\) localization through the entire time period monitored (i.e. 30 min post-stimulation) (Fig. 4B). The stable plasma membrane localiza-
FIGURE 4. Colocalization of B₂R and G protein subunits in HEK293 cells. A, colocalization of B₂R-eYFP (green) and eCFP-Gαₐ (top, red) or Gβγ-eCFP (bottom, red) in a living HEK293 cell in the unstimulated state. B, redistribution of B₂R-eYFP (green) and eCFP-Gαₐ (red) or Gβγ-eCFP (red) in HEK293 cells upon stimulation and receptor internalization.
tion of Gαq after receptor stimulation directly contrasts the behavior of Gαq which internalizes upon cell stimulation (13).

To determine whether B2R is physically associated with G protein subunits in the basal state, we measured the amount of FRET from Gαq-eCFP donors or eCFP-Gβγ donors to B2R-eYFP acceptors in live cells. For the eCFP-eYFP donor-acceptor FRET pair, the distance at which 50% of the donor fluorescence is lost to transfer to the acceptor is 45–50 Å (30). FRET depends on the sixth power of the intermolecular distance between donor and acceptor, therefore, FRET will only occur if these probes are within a close distance (31). In good agreement with the colocalization results described above, we find that both Gαq-eCFP and eCFP-Gβγ transfer ~50% of their emission energy to B2R-eYFP suggesting a high degree of complexation between Gαq and B2R (see “Discussion”). This value can be compared with the 62 ± 3% value for a positive control, eCFP-(GS)₃-eYFP. It is notable that the percentage of FRET from both G protein subunits to B2R is similar and unchanged over a 5-min period (Fig. 5, A and B). Because the percent of FRET from eCFP-Gβγ to B2R-eYFP pair is high as compared with the positive control, and similar to level of FRET from Gαq-eCFP to B2R-eYFP, then these data strongly suggest that B2R, Gβγ, and Gαq are pre-coupled in unstimulated cells.

We monitored the temporal changes in FRET between eCFP-Gαq and eYFP-B2R, and between Gβγ-eCFP to B2R-eYFP upon stimulation with 1 μM bradykinin. Energy transfer for both pairs decreased from 50 to below 20% in the first 3 min and can be compared with 18 ± 1.7% (n = 12) for a negative control (noninteracting membrane bound, eYFP-PLCδ1 and eCFP-Gαq see Ref. 32). This “nonspecific” FRET signal is because of our inability to optically filter bleed through fluorescence completely and normalized FRET values below 20% are out of our range of detection and considered to represent non-interacting species (32). Thus, our results indicate that B2R separates from and Gβγ and Gαq after activation (Fig. 5, A and B).

We have previously found that Gαq remains bound to the plasma membrane after acetylcholine stimulation (32). This result, along with the above studies, suggests that in the basal state G protein subunits are bound to B2R, and upon stimulation they remain on the plasma membrane, whereas B2R

FIGURE 5. In vivo FRET between B2R-eYFP and G protein subunits labeled with eCFP. A, change in the normalized FRET between B2R-eYFP and eCFP-Gαq; B, B2R-eYFP and Gβγ-eCFP upon stimulation with 1 μM BK in cotransfected HEK293 cells. The error bars represent mean ± S.E. of variation between the different trials, where n is the number of cells. These studies were carried out at least four times in independent transfections.
oses from cells that have been transfected with B2R. The partition coefficient of CPM prepared from wild type HEK293 cells is identical to those prepared from natural membranes prepared from either wild type HEK293 cells or transiently transfected cells expressing B2R. The partition coefficient refers to the membrane concentrations at which 50% of the protein is bound and is obtained by fitting the association curve to an exponential rise (see Ref. 21).

We note that the identical concentrations of the B2R-expressing membranes and wild type membranes, as determined by “Experimental Procedures.” FCS follows the fluctuation of the fluorescence in a small and open detection volume. Molecular diffusion is related to this fluctuation. Analysis of the temporal autocorrelation functions (ACF, mathematically expressed as $G(\tau)$) computed from the fluorescence fluctuation data can yield diffusion coefficient of fluorescent molecules. We carried out multiple FCS measurements at room temperature on different transfected HEK293 cells. The autocorrelation function $G(\tau)$ obtained from single point FCS data for B2R-GFP (Fig. 7) best fit to two diffusion coefficients as follows: a fast diffusion component ($2 \times 10^{-7}$ cm$^2$/s), which is attributed to autofluorescence (see “Discussion”), and a slower one ($3.5 \pm 0.6 \times 10^{-9}$ cm$^2$/s, $n = 6$) corresponding to movement of B2R-GFP in the plasma membrane. Single point FCS using 2-photon excitation matched the slower diffusion value of $2.5 \pm 0.6 \times 10^{-9}$ cm$^2$/s ($n = 7$). Some measurements also showed an additional slow diffusion of $4 \times 10^{-10}$ cm$^2$/s suggesting that a portion of the B2R is contained in larger assemblies.

We used scanning FCS to better characterize the slower receptor diffusion in HEK293 cells. In this method, FCS data are simultaneously acquired at multiple points (in this study, 64 points) as the laser beam is moved around an orbit. The orbit crosses the plasma membrane at known points, which allows us to distinguish movement of B2R-GFP in the membrane from movement of the entire cell. Here we used a scanning rate that becomes internalized. This result implies that the strong interactions between G protein subunits and receptor diminish upon activation. To determine whether this is the case, we measured the binding affinity of Gβγ subunits to natural membranes prepared from either wild type HEK293 cells or transiently transfected cells expressing B2R. Binding studies were carried out by covalently attaching a fluorescent probe (CPM) to the G protein subunits and measuring the change in fluorescence intensity as natural membranes are added (Fig. 6).

We note that the identical concentrations of the B2R-expressing membranes and wild type membranes, as determined by phosphate analysis, were used for the titration. We find that both G protein subunits bind strongly to the membranes and that the strength of these interactions is unaffected by an increased level of B2R. These results show that G protein subunits bind to membranes through their own intrinsic affinity, which correlates well with the observation that they remain on the plasma membrane surface as receptor internalizes.

**Mobility of B2R-GFP in HEK293 Cells**—The above studies present physical evidence that in the basal state B2R is complexed with G protein subunits in living cells. There is increasing evidence that GPCRs form dimers (3), and we wanted to assess the extent that the higher order B2R-G protein complexes exist in cells. To accomplish this, we used single point and scanning FCS to measure the mobility of B2R-GFP in transiently transfected HEK293 cells as described under “Experimental Procedures.” FCS follows the fluctuation of the fluorescence in a small and open detection volume. Molecular diffusion is related to this fluctuation. Analysis of the temporal autocorrelation functions (ACF, mathematically expressed as $G(\tau)$) computed from the fluorescence fluctuation data can yield diffusion coefficient of fluorescent molecules. We carried out multiple FCS measurements at room temperature on different transfected HEK293 cells. The autocorrelation function $G(\tau)$ obtained from single point FCS data for B2R-GFP (Fig. 7) best fit to two diffusion coefficients as follows: a fast diffusion component ($2 \times 10^{-7}$ cm$^2$/s), which is attributed to autofluorescence (see “Discussion”), and a slower one ($3.5 \pm 0.6 \times 10^{-9}$ cm$^2$/s, $n = 6$) corresponding to movement of B2R-GFP in the plasma membrane. Single point FCS using 2-photon excitation matched the slower diffusion value of $2.5 \pm 0.6 \times 10^{-9}$ cm$^2$/s ($n = 7$). Some measurements also showed an additional slow diffusion of $4 \times 10^{-10}$ cm$^2$/s suggesting that a portion of the receptor is contained in larger assemblies.

We used scanning FCS to better characterize the slower receptor diffusion in HEK293 cells. In this method, FCS data are simultaneously acquired at multiple points (in this study, 64 points) as the laser beam is moved around an orbit. The orbit crosses the plasma membrane at known points, which allows us to distinguish movement of B2R-GFP in the membrane from movement of the entire cell. Here we used a scanning rate that
was 1 kHz, and so diffusion rates faster than $5 \times 10^{-7}$ cm$^2$/s cannot be resolved.

Results for a representative scanning FCS study are shown in Fig. 8. In Fig. 8A we present a fluorescence image of B$_2$R-GFP in a HEK293 cell where the red circle denotes the scanning path of the laser beam (i.e. the beam orbit). The adjacent intensity carpet (Fig. 8B) shows the intensity traces (all 64 of them) as a function of time. The intensity fluctuations during the measurement at any particular point on the orbit can be found by extracting the appropriate column of interest (e.g. 15 and 42 from Fig. 8B). The intensity fluctuations during the measurement at any particular point on the orbit can be found by extracting the appropriate column from the carpet. Shown in Fig. 8, C and D, are ACFs calculated for columns in the FCS carpet that belongs to the membrane region. The fit of the data to a two-component diffusion equation is in red.

In Fig. 9 we present another example of scanning FCS experiments where we can see very slow movement of B$_2$R-GFP complexes. Fig. 9A shows a cell expressing B$_2$R-GFP and the scanning orbit (red). Intensity carpet calculated from this measurement is presented in Fig. 9B. The carpet shows bright structures along the membrane (position 5 and 28). Fig. 9C shows the same intensity carpet after running it through vertical edge finder routine in SIMFCS (see "Experimental Procedures"). The results show the presence of brighter structures...
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TABLE 1
Summary of the mobility of B2R-GFP measured using different fluctuation techniques
All D values are in cm²/s.

| Technique               | Measured D values | Possible interpretation                                                                 |
|-------------------------|-------------------|----------------------------------------------------------------------------------------|
| 1-Photon excitation     | 2 × 10⁻¹⁰         | Cellular autofluorescence                                                               |
| Single point FCS        | 3.5 ± 0.6 × 10⁻¹⁰ | B2R-GFP freely diffusing in membrane                                                   |
|                         | −4 × 10⁻¹⁰        | Slower moving B2R-GFP diffusing in membrane either as part of larger clusters or bound to cellular structures |
| 2-Photon excitation     | 2.5 ± 0.6 × 10⁻⁹  | B2R-GFP freely diffusing in membrane                                                   |
| Single point FCS        | −3 × 10⁻¹⁰        | Slower moving B2R-GFP diffusing in membrane either as part of larger clusters or bound to cellular structures |
| 2-Photon excitation     | 3.5 ± 1.6 × 10⁻⁹  | B2R-GFP freely diffusing in membrane                                                   |
| Scanning FCS            | 2.9 ± 1.6 × 10⁻¹⁰ | Slower moving B2R-GFP diffusing in membrane either as part of larger clusters or bound to cellular structures |
|                         | 2 ± 1 × 10⁻¹¹     | Larger and brighter structures that show a diffusion coefficient of 2 ± 1 × 10⁻¹¹ may indicate the presence of membrane domains containing many receptors and other protein partners |

FIGURE 9. Intensity profile of diffusing domain structures containing B2R-GFP recorded using scanning FCS with 2-photon excitation. A, fluorescence image of HEK293 cells expressing B2R-GFP recorded with 2-photon excitation. The red circle in the image denotes the path scanned by the laser beam (i.e., the beam orbit) with numbered positions. B, scanning FCS intensity carpet plotted for data recorded from the experiment. C, scanning FCS carpet modified with the vertical edge finder routine in SIMFCS (see “Experimental Procedures”). Brighter structures moving on the membrane are shown with red arrows. D, fluorescence intensity time trace for positions 28 and 50 on the scanning orbit. The profiles were obtained from the scanning FCS carpets shown in B.

We first established the expressed proteins are functional and that the observed B2R-GFP protein complexes are not due to high concentrations of overexpressed protein. The affinity between unactivated Gαq and Gβγ subunits on the surfaces of model membranes has been reported to be 0.1–1 nM depending on the amount of lipid present (33). Additionally, we find that the apparent affinity between purified Gβγ and B2R-GFP in the presence of competing proteins is 50 nM. All of these affinities are 2–3 orders of magnitude greater than our estimated cellular concentration of endogenous and overexpressed proteins, suggesting that overexpression does not trigger the observed protein-protein associations. Interestingly, our Ca²⁺ release studies show that overexpression of B2R lowers cell stimulation of muscarinic receptors suggesting that high expression levels of a particular GPCR may sequester a population of G proteins.

We could not detect any endogenous B2R in HEK293 cells, but we assume that the plasma membrane localization of the fluorescent-tagged protein is appropriate. Alternatively, we (Fig. 9C, indicated by red arrows) that have very long residence time in the observation volume (tens of seconds). From the intensity carpet, we extracted two columns of intensity measurements made at two different positions in the cell, point 28 and 50. Point 28 is on the membrane and point 50 is inside the cell. These intensity time traces are shown in Fig. 9D for the control region on the image (point 50, green line) and for the cell membrane with B2R-GFP expressed in it (point 28, red line). The intensity traces on the membrane show long lived events that are significantly brighter. We note that these results are seen only in 16% of the scanning FCS measurements and represent slow moving structures taking ~10 s to diffuse through the FCS observation volume. These events can give rise to the very slow diffusion coefficients that we measure using scanning FCS techniques.

DISCUSSION

In this study, we present physical evidence showing that B2R exists in complexes with its corresponding Gαq/Gβγ in the basal state of HEK293 cells. These complexes exist at low levels of overexpression of protein and in the presence of competing GPCRs such as acetylcholine receptors. FCS data show these complexes are dynamic and they form higher order structures. Importantly, our results show that signaling through GPCRs involves pre-formed receptor-G protein complexes that are limited by the number of molecules in the complex and allow for rapid, directed signaling.
also note that the plasma membrane localizations of the tagged G protein subunits are identical to that of the endogenous proteins as determined by immunofluorescence. Diffusion of the fluorescently tagged G protein subunits is distinct from the diffusion of cytoplasmic eYFP and bears a resemblance to protein diffusion on membrane. This result argues that the attachment of the fluorescent tag does not perturb the cellular localization of these proteins.

Our FRET studies show that B2R, Gβγ, and Gαq are associated in the basal state of HEK293 cells. FRET measurements were done by attaching eCFP donors to either Gαq or Gβγ and eYFP acceptors attached to B2R. For the eCFP/eYFP pair, it has been reported that the distance at which 50% of the donor emission is lost to acceptors (i.e., R0) is ~50 Å (30, 34). Because FRET efficiency is inversely proportional to the sixth power of eCFP/eYFP distance, a small increase in intermolecular distance could drastically reduce the amount of FRET. Considering the short distance required for FRET is on the order of the sizes of the proteins involved, our results argue that the receptor and G proteins are pre-coupled in the basal state. It is important to note that the measured FRET values have obtained need to be directly compared with control samples measured under identical optical conditions and identical analysis (i.e., 63 and 18%, see below). The values of FRET were obtained using established methods (22, 32) but may have small variations because of photobleaching corrections, etc. The key factor is to assess the changes in FRET for samples that can be directly compared with control samples measured under identical conditions.

G protein subunits are involved in many different signal transduction pathways through different receptors and effectors. In live cells, all G proteins, endogenous and eCFP-tagged, are linked to many different protein partners and cannot be recruited to work with a specific receptor, in this case B2R-eYFP. The possibility of multiple partners will lower the value of normalized FRET between eCFP-tagged G protein subunit and B2R-eYFP. Therefore, ~50% value of normalized FRET between G proteins and B2R receptor indicates that a considerable fraction of Gαq/Gβγ is pre-coupled to B2R receptor.

We find the amount of FRET between eCFP-Gαq and B2R-eYFP, eCFP-Gβγ and B2R-eYFP, and eCFP-Gαq and eYFP-Gβγ all yield a similar value of FRET. This value can be compared with 63% obtained for a positive control in which eCFP and eYFP are linked to a 10-residue flexible chain. Because endogenous G proteins compete for B2R-eYFP, and because endogenous GPCRs compete for eCFP-tagged G protein subunits, our FRET studies show that a substantial amount of the receptor and G proteins is complexes together. These results correlate well with recent studies showing that GIRK channel activation involves movement of pre-formed G protein-channel complexes (35). These observations are also in accord with functional studies showing that Gβγ plays an important role in the GPCR-Gαq activation cycle (28, 29).

Stimulation of cells expressing B2R-GFP with bradykinin resulted in a time-dependent internalization of the B2R-GFP fluorescence. The emergence of a punctate pattern of fluorescence in the plasma membrane was observed as early as 1 min after agonist treatment and was complete by 10 min. We find that B2R-GFP-labeled vesicles were internalized through early endosomes to only a maximum distance of 2 μm. Internalization lasts for a period of at least 45 min in the continued presence of agonist. Taken together, our results suggest that B2R adopts an internalization pathway similar to that of β1AR, which is through small clathrin-independent cytoplasmic vesicles close to the plasma membrane, and different from that of β2AR, which internalizes through a clathrin-dependent mechanism via large perinuclear vesicles (36).

Upon cell stimulation, we observe a time-dependent drop in FRET from Gαq-eCFP to B2R-eYFP and from eCFP-Gβγ to B2R-eYFP from 50 to 20%, which is at our level of detection. This demonstrates that as B2R is internalized, it separates from Gαq and Gβγ subunits, and previous studies showed that Gαq remains on the plasma membrane after acetylcholine stimulation (32). This result contrasts subcellular fractionation studies, which have found that Gβγ translocates from the plasma membrane to soluble fraction upon stimulation of β2AR (37, 38). Additionally, live cell studies have shown that Gβγ subunits associated with Gαq are internalized through a clathrin-independent pathway along with Gαq after β2AR activation (13). This same study has shown combined internalization and possible recycling of Gαq and Gβγ together through Rab11-labeled recycling vesicles. This differing behavior between Gαq/Gβγ movement in response to β2AR stimulation and Gαq/Gβγ movement in response to B2R stimulation may result from the relative affinities of the two types of Gα subunits. We have previously shown that Gαq binds much more weakly to membranes than Gαq (21). We show here that Gαq is not recruited to the membrane by receptor and that membrane association of Gαq is through its own strong intrinsic binding constant. The weaker membrane interactions of Gαq, especially if combined with strong receptor interactions, may allow this protein to internalize more readily.

We used FCS to determine the mobility of the B2R complexes in living cells to assess the distribution of sizes of receptor G protein complexes. Single point FCS measured a diffusion coefficient value of ~3 × 10^-9 cm^2/s for most experiments. Scanning FCS experiments measured three different diffusion coefficient values as follows: 2.9 ± 1.6 × 10^-10 cm^2/s (72%), 3.5 ± 1.6 × 10^-9 cm^2/s (12%), and 2 ± 1 × 10^-11 cm^2/s (16%). Diffusion coefficients of proteins on plasma membrane are in the order of 10^-9 cm^2/s when they are not partitioned into domains or not part of a molecular assembly. Henis et al. (39) found the lateral mobility of β-receptors in membranes of cultured liver cells to be 1.4 ± 0.1 × 10^-7 cm^2/s, and Barak et al. (40) found the mobility of β2AR diffusion coefficient to be 4.0–12 × 10^-9 cm^2/s. An extensive FRAP study on a variety of different membrane proteins expressed in different cell lines report that for transmembrane proteins the diffusion coefficient is ~2 × 10^-9 cm^2/s (41). Recent FCS measurements on live cells show diffusion of β2AR to be 1.01 ± 0.46 × 10^-9 cm^2/s for A549 cells (42), and diffusion coefficient for A1-adenosine receptor Topaz fusion protein, A1-AR-Tpz, diffusion coefficient is reported as 4.27 × 10^-9 cm^2/s (43).
If B$_2$R is bound to a single G protein, the mobility of the complex is not expected to be altered drastically. If receptors and several G proteins form a larger molecular assembly or partition into membrane micro-domains, the average mobility of the receptor would slow down significantly. We believe the slower diffusion (10$^{-10}$ cm$^2$/s and slower) indicates large molecular assembly on the membrane. These slow diffusion coefficients that we measure are biological and not an artifact of the measurement. Similar observations are reported from several other groups (44, 45). Almost always the interpretations of a slower diffusion coefficient are that the receptors form protein clusters or, alternatively, that they are not freely diffusing in the cell membrane but are more closely attached to a cellular structure, e.g. the cytoskeleton.

The cause of the faster diffusion coefficient (2 × 10$^{-7}$ cm$^2$/s) is not completely clear. This component is present in all FCS experiments, and its diffusion coefficient value matches diffusion of a small molecule or protein (i.e. GFP) in cytoplasm. We could not find evidence for free GFP or its analogs in transfected cells because the free fluorophores are small and pass through the nuclear pore, and fluorescence emanating from the nucleus was never observed. A possible explanation for this component would be cellular autofluorescence. Autofluorescence is often considered to be one of the major problems encountered in FCS, especially in 1-photon excitation FCS at wavelength ranges below 500 nm (46, 47). NADH, flavins such as FAD, FMN, and flavoproteins, collagen, and elastin are intrinsic biological autofluorescent molecules, and their fluorescence is detectable under the same filter set used for the reported FCS measurement. Furthermore, for studies on membranes, confocal and also two-photon FCS has the imminent problem that the membrane itself (thickness <10 nm) covers only a vanishing axial fraction of the observation volume. As a consequence, the background of fluorescent molecules in the cytoplasm compromises the observation of membrane-bound molecules.

Autofluorescence studies were carried out in HEK293 cells with 488 nm excitation (data not shown). Intracellular measurements show a correlated background; however, almost no additional correlation was observed at the cell surfaces. The measured intracellular correlation curves from autofluorescence can be described by three-dimensional particle diffusion with typically more than one diffusing species, which is similar to the findings of others (46, 47). The average diffusion coefficients are 10$^{-7}$ cm$^2$/s at different positions in the cell. However, the total fluorescence obtained from autofluorescence is 8–10 times lower, at the same laser power, compared with that obtained from HEK293 cell when receptor is expressed in the plasma membrane. Even though autofluorescence most likely underlies the fast diffusion, we note that GFP is much brighter than these intrinsic fluorophores and therefore its signal dominates in the correlation function.

According to a diffusion model for receptor-G protein signaling, receptor-effector interactions at the plasma membrane are controlled by lateral mobility of the interacting components (2). In contrast to this model, we find here that receptors and G proteins form a spatiotemporally organized system in a confined receptor-G protein stoichiometry rather than a freely diffusible system. Interestingly, our FCS studies show that ~72% of the receptors associate to higher order aggregates. As argued above, these higher order complexes are not expected to be due to protein overexpression. There is an abundance of pharmacological and physical studies, including bioluminescence resonance energy transfer and FRET of other GPCRs, suggesting that these proteins exist as dimers in their native environment (3). Here, our FCS data show that B$_2$R may further associate and thus form higher order signaling domains on the plasma membrane. This result can be correlated to the observation that the main effector of G$_{q/11}$ phospholipase C$eta$, is stably complexed to G$_{q/11}$ in the basal state of PC12 and HEK293 cells (32). Taken together, these results lead to a model in which receptor, G protein, and effector exist in pre-formed complexes involving at least two sets of receptor-G protein-effectors. These domains are thus poised to engage in rapid signaling and give rise to a localized, targeted signal along a unique pathway. Furthermore, these data suggest that intracellular delocalization of the signal occurs primarily through the generation of small and rapidly diffusing second messengers generated from effector activations, such as Ca$^{2+}$ and cAMP, rather than the slower diffusion of the receptor-G protein-effector complexes.

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