The Gi/o protein family transduces signals from a diverse group of G protein-coupled receptors (GPCRs). The observed specificity of Gi/o-GPCR coupling and the high rate of Gi/o signal transduction have been hypothesized to be enabled by the existence of stable associates between Gi/o proteins and their cognate GPCRs in the inactive state (Gi/o-GPCR preassembly). To test this hypothesis, we applied the recently developed technique of two-photon polarization microscopy (2PPM) to Gi1 subunits labeled with fluorescent proteins and four GPCRs: the α2A-adrenergic receptor, GABA_B, cannabinoid receptor type 1 (CB1R), and dopamine receptor type 2. Our experiments with non-dissociating mutants of fluorescently labeled Gi1 subunits (exhibiting impaired dissociation from activated GPCRs) showed that 2PPM is capable of detecting GPCR-G protein interactions. 2PPM experiments with non-mutated fluorescently labeled Gi1 subunits and α2A-adrenergic receptor, GABA_B, or dopamine receptor type 2 receptors did not reveal any interaction between the Gi1 protein and the non-stimulated GPCRs. In contrast, non-stimulated CB1R exhibited an interaction with the Gi1 protein. Further experiments revealed that this interaction is caused solely by CB1R basal activity; no preassembly between CB1R and the Gi1 protein could be observed. Our results demonstrate that four diverse GPCRs do not preassemble with non-active Gi1. However, we also show that basal GPCR activity allows interactions between non-stimulated GPCRs and Gi1 (basal coupling). These findings suggest that Gi1 interacts only with active GPCRs and that the well known high speed of GPCR signal transduction does not require preassembly between G proteins and GPCRs.

G protein-coupled receptors (GPCRs) transduce signals from a variety of extracellular stimuli, such as hormones, neurotransmitters, odorants, tastants, or light, into cells. The primary interacting partners of GPCRs inside cells are heteromeric G proteins, which serve as signal transducers, amplifiers, and modulators (1). Although many aspects of structural and functional interactions between GPCRs and G proteins have been elucidated in recent years, some of the spatiotemporal aspects of these interactions are still not clearly understood.

One of the outstanding issues has been the nature and extent of interactions between non-activated GPCRs and G proteins. Initially (2), non-activated GPCRs and G proteins were thought to not interact and to freely diffuse in the cell membrane. Activation of GPCRs was thought to lead to conversion of the receptors into a form capable of interacting with G proteins, and this transient interaction was thought to occur by collision coupling. However, although a typical cell expresses more than 100 different GPCRs (3), the rates of G protein activation are characterized by rate constants of 30–50 ms (4), which suggests that GPCRs and G proteins might, in fact, interact already prior to GPCR activation (5, 6). Thus, formation of stable complexes between inactive GPCRs and G proteins, termed GPCR-G protein precoupling (5) or preassembly (7, 8), has been postulated. GPCR-G protein preassembly seemed to account for the observed specificity and temporal dynamics of GPCR interactions with G proteins (9).

However, to date, experimental studies of GPCR-G protein coupling have yielded conflicting results, failing to provide consistent evidence supporting either preassembly or collision coupling (10). The mode of GPCR-G protein coupling has been extensively studied in live cells using FRET and bioluminescence resonance energy transfer (BRET) techniques. Preassembly of G proteins to GPCRs in live cells has been suggested based on FRET and BRET data for the Gi1 (11, 12), Gq (13), and Gi/o protein families (8, 12, 14, 15). In contrast, several other FRET studies yielded results consistent with collision coupling of GPCRs to G proteins, particularly for the Gi/o proteins (16, 17). The results are further complicated by the fact that resonance energy transfer signal depends on mutual orientation of fluoroscence moieties, indicates spatial proximity of molecules rather than their physical interaction, and requires two fluorescently modified constructs, which may exhibit non-physiological effects.

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The G protein Gi1 exhibits basal coupling but not preassembly with G protein-coupled receptors

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logical interactions. An alternative approach, relying on fluorescence recovery after photobleaching (FRAP), suggested GPCR-G protein precoupling for the Gα protein (7) but not Gβγ proteins (18). FRAP experiments allow detection of interactions between molecules but require cross-linking of receptor molecules, which might considerably affect the mode of GPCR-G protein coupling. Both resonance energy transfer and FRAP results are affected by protein overexpression in experimental systems, which might lead to overestimation of the extent of GPCR-G protein preassembly (9). Therefore, the issue of G protein-GPCR precoupling remains to be conclusively settled (19).

Another issue complicating interpretation of published experiments is GPCR basal activity. Some GPCRs have been shown to possess constitutive activity (20), which may lead to formation of transient complexes between agonist-free GPCRs and G proteins, resulting in activation of the latter. This interaction, termed "basal coupling" here, is similar to the interaction between the activated GPCRs and G proteins because it is dependent on the GPCR adopting an activated conformation. In contrast, GPCR-G protein preassembly is not caused by the basal activity of the receptor and does not lead to immediate G protein activation (7). Preassembly, unlike basal coupling, increases the rate of signal transfer between stimulated GPCRs and G proteins and thus considerably affects the dynamics of G protein signal transduction.

It should be possible to address the issue of GPCR-G protein preassembly in a new, more conclusive fashion, by using the technique of two-photon polarization microscopy (2PPM), recently developed by our lab (21). In 2PPM, two perpendicular polarizations of the excitation beam are used to acquire two separate images of the sample fluorescence. Differences between the two images (termed linear dichroism (LD)) are indicative of a bias in molecular orientation of the observed fluorophores. Such orientational bias is often present in molecules of fluorescent proteins (FPs) attached to membrane protein molecules. Thus, LD can be observed in most FP-bearing membrane protein constructs. Changes in the LD of the FP labels, indicative of changes in fluorophore orientation, can be used for sensitive observations of changes in protein-protein interactions (such as during G protein activation (22)) or conformational changes in membrane proteins (such as in response to changes in intracellular calcium concentration (21) or cell membrane voltage (23)). Importantly, because of its reliance on only a single fluorescent label, 2PPM allows observations of membrane protein processes at conditions closer to natural than those allowed by resonant energy transfer imaging techniques that rely on two optically active moieties. Furthermore, 2PPM provides information on physical interaction between molecules, not just spatial proximity between molecules. Finally, being a distinct imaging modality, 2PPM can yield distinct structural insights not available by using other imaging techniques, such as FRET (22).

We have now applied 2PPM to observations of molecular interactions between GPCRs of class A (α2AR, CB1R, and D2R) and class C (GABAB) and the G protein Gαi, of the Gαi/o family. We have found that 2PPM allows robust detection of G protein-GPCR interactions. However, we detected only limited interactions between the Gαi protein and non-activated α2AR, GABAβ, or D2R receptors. In contrast, 2PPM experiments with CB1R showed considerable basal coupling but no preassembly of CB1R and Gαi. Thus, our data show that the mode of Gαi/o protein-GPCR interaction is distinct for different GPCRs.

Results

Constructs

To carry out microscopy observations, we obtained several DNA constructs encoding G proteins and GPCRs. Most Gαi constructs were obtained from other laboratories (24, 25). Constructs encoding fluorescently labeled G proteins of two designs (GAP43-CFP-Gαi and Gαi-L91-YFP) were used for the current study, because they had previously been shown to possess functional activity (24, 25) and suitability for 2PPM observations of G protein activation (21, 22). Importantly, these designs of constructs had also been used by others for studying GPCR-G protein interactions using FRET, with contradictory results (12, 16).

To create a positive control for observing GPCR/G protein interactions we created non-dissociating (ND) mutants of fluo-

rescently labeled Gαi constructs. These mutants were made by introducing the N269D mutation, previously shown to impair G protein dissociation from activated GPCRs (25) in yeast (Gpa1 (26)) and mammalian G proteins (Gαi1 (16) and Gαi2 (26)). Both newly created ND constructs (GAP43-CFP-Gαi1(N269D) and Gαi1(N269D)-L91-YFP) show proper membrane localization. Interestingly, even in absence of over-expressed GPCRs, the ND mutants exhibit LD distinct from non-mutated FP-labeled Gαi1 subunits, in both the presence and the absence of Giβγ, indicative of a Gαi1 conformation in the ND mutants distinct from the non-mutated subunits. Our 2PPM measurements also show that the ND mutants of the Gαi1 subunits still bind Gβγ dimers (Fig. 1).

2PPM allows detection of interactions between G proteins and GPCRs

To establish the ability of 2PPM to detect GPCR-G protein interactions, we made 2PPM observations of ND mutants of
FP-labeled G proteins. Because ND mutants of Gα11-FP show impaired dissociation from activated GPCRs, if 2PPM can detect GPCR-G protein interactions, then the Gα11(ND)-FP constructs should exhibit distinct values of LD in the absence and presence of the activated GPCRs. To test this, we transfected HEK293 cells with Gα11(ND)-FP together with Gβ1, Gγ2 and a GPCR (α2A-AR-FP, α2A-AR, GABAB-Snifit (27), CB1R-FP (28, 29), or D2R), stimulated the receptor with an appropriate agonist, and carried out 2PPM observations. The results of these observations are summarized in Fig. 2.

Briefly, the presence of activated GPCRs caused a significant change in LD values of each Gα11(ND)-FP construct. In the GAP43-CFP-Gα11(N269D) construct, the extent of LD, expressed as log2(rmax/rmax0), increased by ~0.5 in the presence of the different activated GPCRs tested (Fig. 2). In the Gα11(N269D)-L91-YFP construct, the value of log2(rmax/rmax0) was higher by ~0.6 in the presence of activated GPCRs. In both Gα11(ND)-FP constructs, small but statistically significant differences in LD were observed between experiments with individual GPCRs, indicating that Gi1 adopts distinct conformations upon interaction with distinct GPCRs. Interestingly, the LD of both Gα11(ND)-FP mutants upon GPCR stimulation was strikingly different from that observed previously in non-mutated Gα11-FP constructs activated by a GPCR (22). This result...
is indicative of \( \alpha_{G1s}^{(ND)} \)-FP being in a state distinct both from a non-activated G protein heterotrimer and an activated (free) \( \alpha_{G1s} \) subunit, and consistent with the protein being associated with the activated GPCR. Importantly, results obtained with non-modified \( \alpha_{2\alpha}^{AR} \)-AR, \( \alpha_{D2}^{R} \), and GABA\(_B\) (a GABA\(_B\) receptor modified only on its extracellular surface) indicate that the observed GPCR-G protein interactions are not caused by nonspecific binding between FP labels of the proteins. Taken together, these results demonstrate that 2PPM allows observations of interactions between the studied GPCRs and the \( \alpha_{G1s} \) protein. Interestingly, some interactions between the \( \alpha_{G1s}^{(ND)} \)-FP constructs and GPCRs could be observed even without agonist activation of the receptor (Fig. 3). In light of our results described below, these interactions are likely due to basal activity of the investigated GPCRs.

**G\(_1\) interacts with non-stimulated CB\(_1\)R but not with \( \alpha_{2\alpha}^{AR}, \alpha_{D2}^{R}, \) or GABA\(_B\) receptors**

Having established, by observing ND mutants of fluorescently labeled \( \alpha_{G1s} \), that 2PPM can visualize GPCR-G protein interactions, we investigated the interactions between GPCRs and non-mutated, fluorescently labeled \( \alpha_{G1s} \). If the \( \alpha_{G1s} \) protein interacts with a GPCR (in the inactive state), the presence of the receptor should affect the observed LD of the \( \alpha_{G1s}^{(ND)} \)-FP constructs. To test this prediction, we transfected HEK293 cells with a \( \alpha_{G1s} \)-FP construct (GAP43-CFP-\( \alpha_{G1s} \) or \( \alpha_{G1s} \)-L91-YFP) along with \( \alpha_{2\alpha}^{AR} \), \( \alpha_{D2}^{R} \), or D\(_2\)R subunits, with or without \( \alpha_{2\alpha}^{AR}, \alpha_{D2}^{R}, \) or GABA\(_B\) receptors, and subjected the transfected cells to 2PPM. The results of our observations are summarized in Fig. 4.

Our 2PPM experiments indicate that the \( \alpha_{G1s} \) protein exhibits distinct modes of interaction with different non-stimulated GPCRs. Co-expression of \( \alpha_{2\alpha}^{AR} \)-AR-FP or non-labeled \( \alpha_{2\alpha}^{AR} \)-AR did not significantly affect the LD of the \( \alpha_{G1s} \)-FP constructs. Similarly, no significant effect on the LD of the \( \alpha_{G1s} \)-FP constructs was observed upon co-expression of the GABA\(_B\) receptor (32). The results indicate that the \( \alpha_{G1s} \) protein does not interact with non-stimulated \( \alpha_{2\alpha}^{AR}, \alpha_{D2}^{R}, \) and D\(_2\)R receptors to a measurable extent. In contrast, co-expression of CB\(_1\)R strongly affected the LD of the \( \alpha_{G1s} \)-FP constructs. This finding indicates that the \( \alpha_{G1s} \) protein interacts with some, but not all non-stimulated GPCRs.

**G\(_1\) exhibits basal coupling but not preassembly with CB\(_1\)R**

The interaction between non-stimulated CB\(_1\)R and the \( \alpha_{G1s} \) protein observed in our 2PPM measurements could be between the \( \alpha_{G1s} \) protein and either the non-activated form of CB\(_1\)R (GPCR-G protein preassembly) or the activated (because of basal activity) form of the receptor (GPCR-G protein basal coupling). Strong basal activity of CB\(_1\)R has been shown previously and confirmed in our experiments (Fig. 5A). To distinguish between preassembly and basal coupling, we eliminated the basal activity of the CB\(_1\)R, either by application of an inverse agonist or by observing a constitutively inactive mutant of CB\(_1\)R (31), incapable of adopting the activated state conforma-

**Discussion**

Binding between inactive G proteins and GPCRs has been proposed as a possible mechanism responsible for the specificity and fast kinetics of interaction between these proteins upon activation. However, previous studies have yielded conflicting results, particularly for proteins of the \( \alpha_{G1s} \) family (12, 14–16, 18). We have now tried to observe G protein-GPCR interactions using 2PPM, a novel optical microscopy technique.
**G\textsubscript{i}1 exhibits basal coupling but not preassembly with GPCRs**

Figure 4. Interaction of non-mutated G\textsubscript{i}1-FP constructs with inactive GPCRs. A, a graph of log\(_2(\text{r}_{\text{max}}/\text{r}_{\text{max}0})\) of the GAP43-CFP-G\textsubscript{i}1 construct co-expressed with G\textsubscript{\beta1}, G\textsubscript{\gamma2} subunits alone (circles) and with \textalpha_{2A}AR-YFP (squares), \textalpha_{2A}AR (triangles), GABA\textsubscript{A}Snifit (inverted triangles), CB\textsubscript{R}YFP (diamonds), or D\textsubscript{2}R (hexagons). B, same graph as in A but for the G\textsubscript{i}1-L91-YFP construct. Both G\textsubscript{i}1-FP constructs did not show a detectable difference in LD upon co-expression with G\textsubscript{\beta1}, G\textsubscript{\gamma2}, \textalpha_{2A}AR, GABA\textsubscript{A} or D\textsubscript{2}R. Co-expression of CB\textsubscript{R}-FP caused a considerably different LD in both G\textsubscript{i}1-FP constructs. The graphs represent the means ± S.D., *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).

Figure 5. Coupling mode of CB\textsubscript{R} with G\textsubscript{i}1-FP. A, GIRK channel currents in HEK293 cells expressing G\textsubscript{i}1 subunit, co-expressed with G\textsubscript{\beta1}, G\textsubscript{\gamma2}, GIRK1-C-CFP and GIRK4 alone (circles) and with CB\textsubscript{R}-YFP (squares). Strong GIRK channel activity is discernible in the presence of CB\textsubscript{R}-YFP without extracellular stimulation. B, membrane localization of CB\textsubscript{R}(T210A)-FP constructs in the HEK293 cells. Bar, 10 \(\mu\text{m}\). C, graph of log\(_2(\text{r}_{\text{max}}/\text{r}_{\text{max}0})\) of the GAP43-CFP-G\textsubscript{i}1 construct co-expressed with G\textsubscript{\beta1}, G\textsubscript{\gamma2} subunits alone (circles), with non-treated CB\textsubscript{R}-FP (squares), CB\textsubscript{R}-YFP inactivated by the inverse agonist (Rimonabant 10 \(\mu\text{M}\)) (triangles), or constitutively inactive mutant CB\textsubscript{R}(T210A)-YFP (inverted triangles). D, same graph as in C but for the G\textsubscript{i}1-L91-YFP construct. Co-expression of CB\textsubscript{R}-FP affected the LD of both studied G\textsubscript{i}1-FP constructs. This effect was dramatically reduced by inhibition of CB\textsubscript{R}-FP basal activity indicating that the G\textsubscript{i}1-CB\textsubscript{R} interaction was caused by basal coupling. The graphs represent the means ± S.D., *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).
G\textsubscript{11} exhibits basal coupling but not preassembly with GPCRs

that allows detection of protein-protein interactions by measuring changes in orientation of a single FP.

Our experiments on the \(\alpha_{\text{11}}\text{-FP}\) mutants, which form stable complexes with activated GPCRs, show that 2PPM allows detection of G protein-GPCR interactions. Furthermore, these experiments show that two of the studied GPCRs (\(\alpha_{2A}\text{-AR}\) and CB\(R\)) interact with the \(G_{11}\) protein without extracellular receptor stimulation. In the absence of extracellular receptor stimulation, no interaction between other studied GPCRs (GABA\(_{\text{b}}\) and D\(_2\)R receptors) and the \(\alpha_{\text{11}}\text{-FP}\) construct was observed.

In contrast to observations of the \(\alpha_{\text{11}}\text{-FP}\) constructs, our 2PPM measurements performed on non-mutated \(\alpha_{\text{11}}\text{-FP}\) subunits without GPCR stimulation showed \(\alpha_{\text{11}}\text{-FP}\) interactions only with CB\(R\), but not with the \(\alpha_{2A}\text{-AR}\), GABA\(_{\text{b}}\), or D\(_2\)R receptors. Importantly, the statistically significant interactions between CB\(R\) and \(\alpha_{\text{11}}\text{-FP}\) could be eliminated by removal of CB\(R\) basal activity, either by application of an inverse agonist or by an inhibiting mutation. These findings indicate that the interaction between the \(G_{11}\) protein and CB\(R\) is caused solely by basal coupling. Although it is conceivable that preassembly occurs without detectably affecting the orientation of the \(\alpha_{\text{11}}\text{-FP}\) fluorescent label and therefore cannot be detected by 2PPM, we find this possibility physically unlikely. Furthermore, our ability to observe interactions between the examined GPCRs and ND mutants of \(G_i\) constructs argues against it. Assuming that a preassembled complex adopts a structure similar to that of the GPCR and an ND mutant, we cannot exclude the possibility that a small fraction of the present \(G_{11}\) protein is preassembled with the observed GPCRs. However, our experiments place rather stringent limits on the size of this fraction. Based on our data, we estimate that our experiments with overexpressed proteins can reveal preassembly if more than 15% of \(\alpha_{\text{11}}\text{-FP}\) molecules is associated with a GPCR. However, endogenous concentrations of GPCRs and G proteins are considerably lower than concentrations of the overexpressed proteins used in our experiments (as we have shown previously (22)). Thus, we extrapolate that in GPCRs expressed at endogenous levels, the fraction of preassembled GPCR-G protein complexes, if present, consists of less than \(\sim 3\%\) of the GPCR molecules.

Our results seem to contradict the conclusions of several previous studies that reported preassembly of G proteins with the investigated receptors. The observed discrepancies can be explained by differences in sensitivity and specificity of the used experimental techniques, as well as by differences in the studied biological systems. Most of the studies showing preassembly relied on resonant energy transfer techniques (FRET or BRET) for detection of G protein-GPCR interactions. A well known difficulty of these techniques is establishing proper positive and negative controls (6). In fact, BRET between many non-interacting proteins has been shown to occur as long as they are co-localized to the same cellular compartment (33). A lack of negative controls taints a study arguing for preassembly of the GABA\(_{\text{b}}\) receptor and the G\(_i\) protein (15). Insufficient controls may have also affected the results of studies of the \(\alpha_{2A}\text{-AR}\) and D\(_2\)R supporting preassembly (12, 14), whereas other studies found no evidence of preassembly (16, 18). Furthermore, FRET and BRET require two labels whose presence may affect the observed protein-protein interaction. In contrast, the 2PPM technique employed in our experiments relies on only a single fluorescent label, and the observed dependence of GPCR-G protein interactions on GPCR activation is an assurance of specificity of the investigated interactions. Previous studies of CB\(R\) interaction with G\(_{11}\) proteins (30, 34) proposed preassembly between these proteins based on indirect evidence, namely competition of CB\(R\) with other GPCRs for the same pool of G\(\alpha\) subunits. However, the studies were carried out with an excess of CB\(R\) over the endogenously expressed G proteins in the cells. Furthermore, it is not clear which of the proteins of the G\(_{11}\) family were present. Therefore, these findings do not directly indicate the physical interaction between CB\(R\) and G\(_{11}\).

Although we did not find preassembly in the four GPCRs used in our study of the G\(_{11}\) protein, we cannot exclude the possibility that preassembly does occur in other GPCRs coupled to G\(_{11}\) or other families of G proteins (7, 14). Preassembly might depend on the presence of specific motifs in GPCRs, such as the polybasic motif (7) deemed to be needed for preassembly of GPCRs that couple to the G\(_i\) protein (not investigated in this study). Although a small number of G\(_{11}\)-coupled GPCRs do contain the polybasic motif (39), none of them have been reported to date to preassemble with their cognate G proteins. Further research is required to elucidate the role of the polybasic motif in interactions between the GPCRs and G\(_{11}\) proteins. Furthermore, in promiscuous GPCRs, preassembly may also occur specifically with a particular G protein (8). Therefore, we cannot exclude that preassembly occurs in GPCR/G protein signaling systems other than described here. However, G protein activation kinetics by the GPCRs used in this study (e.g. \(\alpha_{2A}\)-AR (16)) are not slower than those of GPCRs shown to be preassembled with G proteins (7, 8). Thus, our results indicate that high specificity and rate of signaling can be achieved without pre-existing physical interaction between a GPCR and a G protein.

Our results point to the importance of GPCR basal activity for GPCR-G protein interactions. Basal activity has been demonstrated for a multitude of GPCRs (20) coupling to different families of G proteins. Therefore, the observed role of basal coupling in GPCR-G protein interactions is likely important for many GPCRs. Our results can be viewed in the context of models proposing spatial focusing (35) of G protein activation in the vicinity of GPCRs with basal activity: transient interactions between G proteins and a spontaneously activated GPCR may enrich the local environment in the vicinity of the GPCR in activated molecules of its cognate G proteins. The effects of a non-stimulated GPCR on cellular metabolism depend on the extent of its basal activity (20), expression levels of the receptor and the cognate G proteins (36), the activity of G protein-inactivating proteins (35), and the diffusion rates of the involved proteins (37). In light of our results, the well known high rate of G protein activation (often cited as evidence for GPCR-G protein preassembly (9)) is likely the result of high G protein concentration in the cell membrane.

In summary, our experiments show that 2PPM allows sensitive detection of interactions between GPCRs and G proteins in...
$G_{α1}$ exhibits basal coupling but not preassembly with GPCRs

living cells. Our results demonstrate that GPCRs can interact with the G protein $G_{α1}$ even without external activation of the receptor. However, this interaction depends on the spontaneous activity of the receptor. Although we have not carried out any direct comparison of our 2PPM results on GPCR-G protein interactions with other techniques, in our previous work on G protein dissociation (22) 2PPM sensitivity was on par with FRET. Importantly, the systems investigated by 2PPM were protein dissociation (22) 2PPM sensitivity was on par with interactions with other techniques, in our previous work on G protein activity of the receptor. Although we have not carried out any direct comparison of our 2PPM results on GPCR-G protein

Materials and methods

Molecular biology

Most of the constructs used in this study were kind gifts from A. Tinker (GAP43-CFP-Gα1), M. Bünemann ($G_{α1}$-L91-YFP, α2AR-AR-CFP, and α2AR-AR-YFP), K. Johnsson (GABA$_A$-Snif containing the GABA$_B_{1a}$ Subunit tagged with Snap and Clip tags and non-labeled GABA$_B_{2}$ subunit), Z. Lenkei (CB$_1$R-CFP and CB$_1$R-YFP), N. Lambert (D$_2$R), V. Ruiz-Velasco (Gβ1 and Gγ2), and E. Reuveny (GIRK1-C-CFP and GIRK4). The N269D mutants of G$_{α11}$-FP constructs and T210A mutants of the CB$_1$R-FP constructs were created by PCR mutagenesis (Phusion polymerase; New England Biolabs) and verified by sequencing (LGC Genomics, Berlin, Germany).

Cell culture

Human embryonic kidney 293 cells (HEK293) were cultured in 25-cm$^2$ flasks in DMEM supplemented with 10% fetal bovine serum in the atmosphere of 5% CO$_2$ at 37 °C. For imaging experiments, the cells were plated on 8-well microscopy slides (µ-Slides; Ibidi, Martinsried, Germany). Transfection was performed using Lipofectamine 2000 (Life Sciences) according to the manufacturer’s protocol. Equimolar amounts of all constructs were used in co-transfections. Transfected cells were incubated for 48 h prior to imaging. For activation experiments, DMEM was replaced with HEPES-buffered Hanks’ balanced salt solution (HHBSS, pH 7.4), and the GPCR ligands norepinephrine ((±)-norepinephrine (+)-bitartrate salt; final concentration, 1 µM; Sigma), GABA (final concentration, 1 mM; Sigma), CP-55940 (final concentration, 2.5 µM; Sigma; Covalys Biosciences, Witterswil, Switzerland) (27) for 30 min in HHBSS at 37 °C before imaging, washed four times with PBS and transferred into fresh HHBSS medium.

Two-photon polarization microscopy

The technique of 2PPM and image analysis procedure are described in detail in Refs. 21 and 22. Briefly, imaging experiments were carried out on a customized Imic2 microscope (Till Photonics, Gräfelfing, Germany) equipped with a titanium:sapphire laser (Chameleon Ultra II with GVD compensation; Coherent, Santa Clara, CA), using a UAp0Plan/IR × 60 NA1.2 water-immersion objective lens (Olympus). A long-pass dichroic mirror and an emission filter (Q565LP (Chroma, Foot hill Ranch, CA) with Brightline 479/40 (Semrock, Rochester, NY) for CFP, 740DCXR (Chroma) with Brightline 542/27 (Semrock) for YFP, and 740DCXR (Chroma) with 620/60 (Chroma) for BG-TMR) separated fluorescence from the excitation laser beam. Fluorescence was detected by a photomultiplier (R6357; Hamamatsu Photonics) equipped with an IR-blocking filter (HQ700SP-2P; Chroma). Excitation light polarization was alternated between horizontal and vertical by a polarization modulator (RPM-2P; Innovative Bioimaging) synchronized with the microscope and operating at 100 kHz. Images were typically acquired at 50 × 100-nm pixel size and 10-µs pixel dwell time. Raw images were deinterleaved into pairs of images showing fluorescence excited with light polarized horizontally and vertically, respectively. The images were processed and quantitatively analyzed as described previously (21, 22). LD was quantitatively expressed in terms of the maximum dichroic ratio ($r_{\max}$) and the logarithm of the $r_{\max}/r_{\max 0}$ ratio ($\log_2(r_{\max}/r_{\max 0})$), where $r_{\max}$ and $r_{\max 0}$ are the maximum dichroic ratio for the tested and reference datasets, respectively (usually the FP-tagged G protein heterotrimer expressed with and without GPCRs). At least 10 cells were quantitatively analyzed for each experimental condition.

Electrophysiology

Recordings of membrane current were performed using the whole-cell patch-clamp technique (38) using an EPC10 USB amplifier (HEKA Elektronik Dr. Schulze, Lambrecht, Germany). Patch pipettes were pulled from borosilicate glass capillaries (GC150T-10; Harvard Apparatus, Holliston, MA) using a PC-10 vertical puller (Narishige, Amityville, NY). Pipette resistances were in the range of 2–6 MOhm when filled with pipette solution. The data were acquired and analyzed using Patchmaster software (HEKA Elektronik Dr. Schulze). $I_{GIRK}$ was measured as an inward current using a holding potential –90 mV. Internal (pipette) solution composition was 100 mM potassium aspartate, 40 mM KCl, 5 mM MgATP, 10 mM HEPES, 5 mM NaCl, 2 mM EGTA, 1 mM MgCl$_2$, pH 7.3. External solution composition was 120 mM NaCl, 20 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.3.

Extrapolation of the extent of G protein preassembly with GPCRs between different concentrations

Extrapolating the extent of G protein preassembly with GPCRs between different concentrations, for a simple binary G protein-GPCR interaction

$$G + R \leftrightarrow GR \quad (\text{Eq. 1})$$

The dissociation constant for this reaction is

$$K_d = \frac{[G][R]}{[GR]} \quad (\text{Eq. 2})$$

Concentration of the overexpressed GPCR is

$$c_{GR} = [R] + [GR] \quad (\text{Eq. 3})$$
Concentration of the overexpressed G protein is

\[ c_{\text{Gx}} = [G] + [\text{GR}] \]  
(Eq. 4)

In overexpression systems \( c_{\text{Gx}} = c_{\text{x}} = c_{\text{G}} \).

If 15% of the GPCR molecules are bound to the G protein

\[ [\text{GR}] = 0.15c_{\text{x}} \]  
(Eq. 5)

Then the dissociation constant is,

\[ K_d = \frac{(c_{\text{x}} - 0.15c_{\text{G}})(c_{\text{x}} - 0.15c_{\text{G}})}{0.15c_{\text{x}}} = 5c_{\text{x}} \]  
(Eq. 6)

Therefore,

\[ c_{\text{x}} = \frac{K_d}{5} \]  
(Eq. 7)

The G protein in our experiments is expressed at a level \(~5\) times higher than endogenous concentration (22).

Endogenous (5 times lower) G protein concentration \( c_{\text{n}} \) will be

\[ c_{\text{n}} = \frac{K_d}{25} \]  
(Eq. 8)

For equal concentrations of the GPCR and the G protein

\[ K_d = \frac{[G][R]}{[\text{GR}]} = \frac{[R][R]}{[G] - [R]} \]  
(Eq. 9)

\[ \frac{K_d^2}{25} - K_d[R] = [R]^2 \]  
(Eq. 10)

\[ [R]^2 + K_d[R] - \frac{K_d^2}{25} = 0 \]  
(Eq. 11)

Concentration of the free GPCR \([R]\) is

\[ [R] = \frac{-K_d + \sqrt{K_d^2 + 4K_d^2/25}}{2} \]  
(Eq. 12)

The fraction of the free GPCR is

\[ \frac{[R]}{c_{\text{n}}} = \frac{-K_d + \sqrt{K_d^2 + 4K_d^2/25}}{2K_d/25} = 0.963 \]  
(Eq. 13)

Hence the fraction of the GPCR in complex is

\[ \frac{[\text{GR}]}{c_{\text{n}}} = 1 - 0.963 = 0.037 \]  
(Eq. 14)

For a GPCR concentration lower than G protein concentration, the fraction in complex will be even lower.

**Statistics**

Statistical significance of the data was evaluated using Student’s \( t \) test for comparison of two groups. When more than two groups were compared, we used one-way analysis of variance followed by the Bonferroni post test comparison. The normality of the data was tested and confirmed by D’Agostino-Pearson Omnibus \( K^2 \) normality test.

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