The GPRC6A receptor displays constitutive internalization and sorting to the slow recycling pathway

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The class C G protein-coupled receptor GPRC6A is a putative nutrient-sensing receptor and represents a possible new drug target in metabolic disorders. However, the specific physiological role of this receptor has yet to be identified, and the mechanisms regulating its activity and cell surface availability also remain enigmatic. In the present study, we investigated the trafficking properties of GPRC6A by use of both a classical antibody feeding internalization assay in which cells were visualized using confocal microscopy and a novel internalization assay that is based on real-time measurements of fluorescence resonance energy transfer. Both assays revealed that GPRC6A predominantly undergoes constitutive internalization, whereas the agonist-induced effects were imperceptible. Moreover, postendocytic sorting was investigated by assessing the co-localization of internalized GPRC6A with selected Rab protein markers. Internalized GPRC6A was mainly co-localized with the early endosome marker Rab5 and the long loop recycling endosome marker Rab11 and to a much lesser extent with the late endosome marker Rab7. This suggests that upon agonist-independent internalization, GPRC6A is recycled via the Rab11-positive slow recycling pathway, which may be responsible for ensuring a persistent pool of GPRC6A receptors at the cell surface despite chronic agonist exposure. Distinct trafficking pathways have been reported for several of the class C receptors, and our results thus substantiate that non-canonical trafficking mechanisms are a common feature for the nutrient-sensing class C family that ensure functional receptors in the cell membrane despite prolonged agonist exposure.

The G protein-coupled receptor, class C, group 6, member A (GPRC6A),2 is a nutrient-sensing G protein-coupled receptor (GPCR) belonging to class C. It is activated by basic l-α-amino acids and divalent cations, which trigger coupling to the Go protein and thereby an increase in the intracellular levels of the second messengers inositol trisphosphate and Ca2+(1–8). Other ligands and signaling pathways have been reported (3, 9–13); however Rueda et al. (8) and we (7) have not been able to confirm these findings. The GPRC6A receptor displays a broad but low expression profile in human, mouse, and rat (2, 5, 10, 11, 14, 15), thus giving little indication to the physiological role of the receptor. Several groups have conducted studies using GPRC6A knock-out mice to elucidate the physiological function of the receptor, and although results differ between knock-out mouse models, they altogether suggest an involvement in metabolism and endocrine regulation (6, 10, 11, 16–18).

Over the years, it has become evident that GPCR signaling is much more complex than once believed. For most receptors, a variety of ligands and intracellular signaling pathways are available, and numerous regulatory mechanisms are thus required for obtaining specific biological responses (19). The process of receptor trafficking plays a critical role in regulating GPCR function by controlling the level of receptors in the cell membrane, hence controlling the number of receptors that are available for activation by extracellular ligands. Receptor trafficking includes the maturation and insertion of newly synthesized receptors in the cell membrane as well as the internalization of receptors from the surface and the subsequent intracellular sorting. Receptor phosphorylation and internalization are of fundamental importance for GPCR signal termination (20).

Despite the prominence of this type of regulation, very little is known about GPRC6A trafficking. It has been demonstrated that GPRC6A undergoes N-glycosylation (21), which is a post-translational modification involved in maturation of receptors and correct surface targeting of mature proteins. Oligomerization is another important mechanism that controls surface targeting and/or function. For class C receptors in particular, homo- or heterodimerization has proven essential for receptor function, and accordingly homodimerization has been demonstrated for the GPRC6A receptor (2, 21). However, the regula-
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To measure internalization of myc-GPRC6A, antibody feeding experiments were conducted in which surface receptors were labeled with a primary anti-myc antibody prior to internalization at 37 °C in buffer ± agonist. Subsequently, the surface-expressed receptors were detected using a secondary antibody conjugated to Alexa Fluor 568, and following cell permeabilization, internalized receptors were detected using a secondary antibody conjugated to Alexa Fluor 488. Cells were then visualized using confocal microscopy. The primary anti-myc antibody displayed very poor labeling at 4 °C compared with 37 °C (Fig. 1A), and the antibody labeling was thus performed at 37 °C. The results showed that, following primary antibody labeling, incubation in buffer alone at 37 °C was sufficient to induce significant endocytosis of GPRC6A (Fig. 1B), thereby indicating that the receptor internalizes constitutively. As a control, myc-tagged β2-adrenergic receptor (β2AR) was also tested. β2AR is a well characterized receptor known to internalize mainly upon agonist stimulation (25); thus it should display very limited constitutive internalization. Correspondingly, myc-β2AR showed little internalization when testing buffer alone (Fig. 1B), and the level of agonist-independent internalization of myc-GPRC6A was significantly higher (Fig. 1C). To preclude that surface receptor labeling at 37 °C was responsible for promoting the observed constitutive internalization of GPRC6A, an HA-tagged version of the receptor was also tested. Surface receptors were successfully labeled with anti-HA antibodies at 4 °C, and the subsequent incubation in buffer at 37 °C gave rise to constitutive internalization of GPRC6A (Fig. 1D) in accordance with results for myc-GPRC6A. Finally, SNAP-tagged GPRC6A was surface-labeled at 4 °C with a cell-impermeable SNAP substrate conjugated to Alexa Fluor 488. Again, buffer incubation at 37 °C allowed constitutive internalization (Fig. 1E), thus verifying that the internalization observed for myc-GPRC6A and HA-GPRC6A was not mediated by the antibodies.

The presence of amino acids and divalent cations in cell culture media and buffers is a major challenge when studying GPRC6A signaling and trafficking. To minimize the influence from these omnipresent agonists, the rat GPRC6A receptor was used in the present study as GPRC6A ligands are less potent for the rat receptor than for mouse and human GPRC6A (5). Nevertheless, receptor agonists are present in the cell culture medium prior to the antibody feeding experiments, and although present in low concentrations, they could be responsible for the observed internalization. When testing recombinant expressed mouse GPRC6A in functional assays, incubation in a washing buffer for 2 × 2 h prior to ligand stimulation is a requirement for establishing agonist responses. The washing protocol is used to remove the omnipresent agonists, thereby reducing basal receptor signaling (4, 26). In the present study, the effect of the washing protocol on functional responses of rat GPRC6A was tested. As rat GPRC6A is less prone to be affected by the omnipresent agonists, the washing protocol was not a requirement; however, functional responses did significantly increase upon introducing a 2-h wash. Washing 2 × 2 h did not increase the response further (Fig. 2A). If omnipresent agonists are mediating the observed internalization of rat GPRC6A, introducing a 2-h wash to remove the agonists should thus decrease the level of basal internalization. However, no decrease in internalization was observed when including a washing protocol in the antibody feeding experiment. On the contrary, a small but significant increase in internalization was detected (Fig. 2, B and C). Measuring the surface and total expression of myc-GPRC6A by enzyme-linked immunosorbent assay (ELISA) further demonstrated that the expression level of GPRC6A was unaffected by the washing protocol (Fig. 2D). This indicates that even if agonists are present in cell culture media and/or buffers they are not responsible for the observed endocytosis of GPRC6A.

To further substantiate that GPRC6A internalization is constitutive, a mutant receptor in which the orthosteric binding site was impaired by mutation was tested. The class C GPCRs share a common orthosteric binding site in which five residues are highly conserved throughout the receptor subtypes activated by L-α-amino acids (i.e. the metabotropic glutamate receptors (mGluRs), GPRC6A, the calcium-sensing receptor (CaSR), and the T1R1 taste receptor) (27, 28). Crystal structures of the mGluRs have proven that these five residues are in fact located in the orthosteric binding site (29–31). Asp-303 in GPRC6A is one of these highly conserved residues, and it corresponds to Asp-301 in mGluR3 and Glu-297 in CaSR. Mutagenesis studies have verified the importance of this specific residue in L-α-amino acid-mediated activation/binding of mGluR3, CaSR, and the goldfish GPRC6A ortholog 5.24 (32–34). CaSR is the closest mammalian homolog of GPRC6A, and because crystal structures of CaSR verify that Glu-297 is located...
in the orthosteric binding site (35, 36), it is reasonable to assume that Asp-303 is also found in the orthosteric binding site of GPRC6A, although no structural information is yet available for this receptor. In the current study, the aspartic acid was thus substituted with alanine (D303A) to impair the agonist-binding site in GPRC6A. Accordingly, D303A showed no functional response to L-ornithine (L-Orn) or Ca\(^{2+}\), and it is thus non-responsive to GPRC6A agonists despite being expressed at the cell surface (Fig. 3, A and B). When tested in the antibody feeding experiment, the mutant showed no significant difference in level of internalization compared with the WT receptor (Fig. 3, C and D), strongly suggesting that the observed internalization of WT GPRC6A is constitutive.

**A novel real-time internalization assay confirmed the constitutive internalization of GPRC6A**

A novel, real-time internalization assay was performed using a SNAP substrate conjugated to Alexa Fluor 488 for 30 min at 4 °C, and constitutive internalization was allowed by 30-min incubation in buffer at 37 °C. Representative images (n = 20) from two independent experiments are shown. In all images, insets show high magnification of the regions indicated by white rectangles. Scale bars, 10 μm.

**Figure 1. GPRC6A displays constitutive internalization.** A, confocal microscopy images of HEK293 cells co-transfected with myc-GPRC6A and GqG66D in a 1:1 ratio. Surface receptors were labeled using primary anti-myc antibody for 30 min at 37 or 4 °C as indicated, and constitutive internalization was allowed for 30 min. Representative images (n = 30) from three independent experiments are shown. B, confocal microscopy images of HEK293 cells co-transfected with myc-GPRC6A and GqG66D in a 1:1 ratio (top panel) or myc-β2AR (bottom panel). Surface receptors were labeled using primary anti-myc antibody for 30 min, and constitutive internalization was allowed by 30-min incubation in buffer, both being performed at 37 °C. Representative images (n = 20) from two independent experiments are shown. C, quantification of B. One cell per image was chosen for quantification of surface labeling and internalized labeling, respectively. Data are shown as internalized/surface ratio normalized to β2AR. 20 cells from two independent experiments were used, and the means are shown with error bars representing S.E. Statistical analysis was performed using an unpaired Student’s t test (***, p < 0.001). D, confocal microscopy images of HEK293 cells co-transfected with HA-GPRC6A and GqG66D in a 1:1 ratio. Surface receptors were labeled using anti-HA antibody for 30 min at 4 °C, and constitutive internalization was allowed by 30-min incubation in buffer at 37 °C. Representative images (n = 30) from three independent experiments are shown. E, confocal microscopy images of HEK293 cells co-transfected with SNAP-GPRC6A and GqG66D in a 1:1 ratio. Surface receptors were labeled using a SNAP substrate conjugated to Alexa Fluor 488 for 30 min at 4 °C, and constitutive internalization was allowed by 30-min incubation in buffer at 37 °C. Representative images (n = 20) from two independent experiments are shown. In all images, insets show high magnification of the regions indicated by white rectangles. Scale bars, 10 μm.
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Figure 2. The effect of removing omnipresent agonists on GPRC6A function, expression, and internalization. HEK293T cells co-transfected with myc-GPRC6A and GqG66D in a 1:1 ratio were tested. A, myc-GPRC6A function was assessed using the IP-One assay. Three conditions were tested: 2 × 2-h wash, 2-h wash, and no wash prior to ligand stimulation. Data are shown as -fold increase over basal IP₁, levels and are means of three independent experiments performed in triplicates. Error bars represent S.E. Statistical analysis was performed using an unpaired Student’s t test between the no-wash and 2-h-wash conditions at highest L-Orn concentration (**, p < 0.001). B, confocal microscopy images of myc-GPRC6A. Surface receptors were labeled using primary anti-myc antibody for 30 min, and constitutive internalization was allowed by 30-min incubation in buffer. Two conditions were tested: with or without a 2-h wash prior to labeling. Insets show high magnification of the regions indicated by white rectangles. Representative images (n = 30) from three independent experiments are shown. Scale bar, 10 μm. C, quantification of B. One cell per image was chosen for quantification of surface labeling and internalized labeling, respectively. Data are shown as internalized/surface ratio normalized to the condition with no wash. 30 cells from three independent experiments were used, and the means are shown with error bars representing S.E. Statistical analysis was performed using an unpaired Student’s t test (*, p < 0.05). D, determination of surface (left) and total (right) expression of myc-GPRC6A by ELISA with or without a 2-h wash prior to PFA fixation. Data are means of three independent experiments performed in triplicates with error bars representing S.E. au, arbitrary light units.

low donor/acceptor ratio, whereas receptor internalization prevents energy transfer to the extracellular acceptor, thus increasing the donor/acceptor ratio. Roed et al. (37) have verified the assay by means of glucagon-like peptide 1 receptor (GLP1R) and β₂AR, both of which were labeled at 37 °C and showed agonist-induced endocytosis. However, when testing receptors that internalize constitutively, it is desirable to prevent trafficking during the labeling by lowering the temperature to 4 °C. In the present study, SNAP-β₂AR was used to verify the real-time internalization assay with labeling being performed at 37 or 4 °C, respectively. Agonist stimulation and real-time measurements were, however, performed at 37 °C in all experiments to allow trafficking. As seen from Fig. 4, A and B, internalization was triggered upon incubation with a 10 μM concentration of the β₂AR agonist isoproterenol (ISO). The level of internalization was unaffected by the labeling conditions, although internalization was delayed following labeling at 4 °C (Fig. 4, A and B). The time required for heating up the plate from 4 to 37 °C in the EnVision plate reader likely accounts for the observed delay in internalization. In conclusion, the novel internalization assay successfully detects agonist-induced internalization following receptor labeling at 4 °C.

SNAP-tagged GPRC6A was also tested in the real-time internalization assay upon labeling being performed at 4 or 37 °C, respectively. In accordance with results from the antibody feeding experiments, data showed that GPRC6A internalized upon incubation in buffer at 37 °C (Fig. 4C). However, labeling at 37 °C resulted in an increased level of internalization already at the start of the measurement (at “0 min” in Fig. 4C), and moreover, the increase in internalization over time was very limited compared with labeling at 4 °C. These results suggest that the labeled receptors had already approached a steady-state internalization/recycling equilibrium during the labeling process at 37 °C, and consequently it was only possible to measure a small increase in internalization. By lowering the labeling temperature to 4 °C, constitutive internalization was prevented during the labeling process, and a more pronounced increase in internalization could thus be measured. In agreement with results described above, introducing a 2-h washing step in the real-time internalization assay triggered, if anything, a minor increase in GPRC6A internalization (Fig. 4D). When testing the SNAP-tagged D303A mutant, constitutive internalization was likewise still detected, although the results indicated that the rate of internalization was slower for D303A. Moreover, the steady-state level of internalization was lower compared with the WT receptor (Fig. 4E); however, this reduction in internalization signal for D303A is likely accounted for by a reduction in overall surface expression as indicated by the ELISA data (Fig. 3B) and a reduced surface labeling of D303A (Fig. 4F). The results confirm altogether that GPRC6A internalizes constitutively and that the real-time
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**Figure 3. The non-functional D303A mutant displays constitutive internalization.** HEK293T cells co-transfected with myc-GPRC6A (WT or D303A) and Gq\textsubscript{G66D} in a 1:1 ratio were tested. A, myc-GPRC6A WT and D303A function was assessed using the IP-One assay. The GPRC6A agonists L-Orn and Ca\textsuperscript{2+} were tested in increasing concentrations. Data are means of a single representative experiment performed in triplicates with error bars representing S.D. Two additional experiments gave similar results. B, determination of surface (left) and total (right) expression of myc-GPRC6A WT and D303A were conducted using ELISA. Data are means of three independent experiments performed in triplicates with error bars representing S.E. C, confocal microscopy images of myc-GPRC6A WT and D303A. Surface receptors were labeled using primary anti-myc antibody for 30 min, and constitutive internalization was allowed by 30-min incubation in buffer. Insets show high magnification of the regions indicated by white rectangles. Representative images (n = 30) from three independent experiments are shown. Scale bar, 10 μm. D, quantification of C. One cell per image was chosen for quantification of surface labeling and internalized labeling, respectively. Data are shown as internalized/surface ratio normalized to WT. A statistical outlier test was performed, and data points identified as outliers were excluded. Thus at least 27 cells from three independent experiments were used, and the means are shown with error bars representing S.E. Statistical analysis was performed using an unpaired Student’s t test (ns, p > 0.05). au, arbitrary light units.

Internalization assay is very useful in detecting constitutive endocytosis if receptor labeling is being performed at 4 °C.

**Co-expression of the Gq\textsubscript{G66D} protein is not mediating constitutive internalization**

When testing mouse GPRC6A in transient mammalian expression systems, it has been necessary to co-express a mutated Gq\textsubscript{G66D} protein that facilitates Gq coupling to obtain robust, functional responses (4, 26, 39). Likewise, no functional response of rat GPRC6A was measured in the absence of Gq\textsubscript{G66D} (Fig. 5A); thus overexpression of this G protein is a prerequisite for detecting activity of transiently expressed GPRC6A in human embryonic kidney (HEK) 293T cells. In the present study, all internalization experiments of GPRC6A so far have been conducted in the presence of Gq\textsubscript{G66D}. When tested in the absence of Gq\textsubscript{G66D}, the receptor still displayed constitutive internalization, although Gq\textsubscript{G66D} did trigger a minor increase in the internalized/surface ratio when using the antibody feeding assay (Fig. 5, B and C). On the contrary, the presence of Gq\textsubscript{G66D} decreased the internalization of GPRC6A when tested in the real-time internalization assay. This effect, however, was minor, whereas the rate of internalization was largely unaffected by the presence of Gq\textsubscript{G66D} (Fig. 5D). Accordingly, GPRC6A displays constitutive internalization in the absence of Gq\textsubscript{G66D}, and the internalization is thus not mediated by the Gq\textsubscript{G66D} protein. To further investigate the influence of Gq\textsubscript{G66D} on constitutive internalization, GPRC6A was measured in the presence of the Gq\textsubscript{G66D}-specific inhibitor.
FR900359 (40, 41). In the functional IP-One assay, the L-Orn-induced response of GPRC6A was completely inhibited by 1 μM FR900359 (Fig. 5E); however, in the real-time internalization assay, the Gq inhibitor had no effect on the level of GPRC6A internalization (Fig. 5F). These results thus further substantiate that the internalization is independent of the activity of GPRC6A.

The GPRC6A receptor does not display constitutive activity

As seen in Fig. 5E, the Gq inhibitor FR900359 is capable of inhibiting the basal level of IP1 for WT GPRC6A, which indicates that the receptor displays constitutive activity. As constitutive activity could be triggering constitutive endocytosis, the basal activity of WT GPRC6A was further investigated. To exclude effects from omnipresent amino acids, the D303A mutant was also included in the studies. No difference in the basal IP1 level was detected among WT, D303A, and the pEGFPN1 vector (Fig. 6), thus indicating no agonist-independent activity of the receptor. To confirm this finding, the constructs were also tested in the presence of FR900359, which should be able to decrease the basal IP1 level if the receptor displays constitutive activity. In accordance with results shown in Fig. 5E, FR900359 did inhibit the basal IP1 production, but the level of inhibition for WT GPRC6A and D303A was not different from that of the pEGFPN1 vector (Fig. 6), and the inhibition is thus likely a result of inhibiting basal activity of other Gq-coupled GPCRs natively expressed in HEK293T cells. Finally, all three constructs were tested while co-expressed with

Figure 4. Detecting receptor internalization using the novel, real-time internalization assay. HEK293T cells co-transfected with SNAP-GPRC6A (WT or D303A) and Gq,G66D in a 1:1 ratio or transfected with SNAP-β2AR were tested as indicated in the figure. A, labeling of SNAP-β2AR was performed at 37 °C. Buffer ± 10 μM ISO was added to allow internalization. B, labeling of SNAP-β2AR was performed at 4 °C. Buffer ± 10 μM ISO was added to allow internalization. C, labeling of SNAP-GPRC6A WT was performed at 37 and 4 °C, respectively. Buffer was added to allow constitutive internalization. D, SNAP-GPRC6A WT was tested with and without a 2-h wash prior to labeling. The labeling was performed at 4 °C, and buffer was added to allow constitutive internalization. E, the SNAP-GPRC6A-D303A mutant was tested. Labeling was performed at 4 °C, and buffer was added to allow constitutive internalization. F, donor emission of SNAP-GPRC6A WT and D303A upon labeling with Tag-lite SNAP Lum4-Tb substrate. No acceptor fluorophore or ligands were added. Donor emission is equivalent to the amount of labeling and hence the amount of surface receptors. All graphs are means of a single representative experiment performed in triplicates with error bars representing S.D. Two additional experiments gave similar results.
GqG66D. The basal level of WT GPRC6A was markedly increased; however, the D303A mutant did not display any increase compared with pEGFPN1 (Fig. 6), and the effect at the WT receptor must therefore arise from increased sensitivity toward omnipresent agonists upon GqG66D co-expression. Collectively, these results show that GPRC6A does not display any constitutive activity.

Agonist stimulation has very limited effect on GPRC6A internalization

To test whether agonist stimulation induced further endocytosis of the GPRC6A receptor, antibody feeding experiments were performed in which internalization was allowed by incubation in buffer alone. Three different conditions were tested: 30 min of buffer alone, 15 min of buffer followed by 15 min of L-Orn, and 30 min of L-Orn. Quantification revealed a minor but significant increase in the internalized/surface ratio after 15 min of L-Orn stimulation (Fig. 7, A and B). However, no difference was detected in the internalized/surface ratio between buffer and 30 min of L-Orn (Fig. 7, A and B). As a positive control, myc-β2AR was tested upon stimulation with 9 μM ISO. Consistent with the results reported above, little internalization was detected upon incubation with buffer alone. However, stimulation with agonist for
Figure 6. GPRC6A does not display constitutive activity. HEK293T cells co-transfected with myc-GPRC6A (WT or D303A) and Gq66D (or pEGFPN1) in a 1:1 ratio were tested as indicated in the figure. The basal level of activity was measured by incubation in ligand buffer in the IP-One assay. The experiment was conducted in the presence and absence of a 1 μM concentration of the Gq6-specific inhibitor FR900359. Data are means of three independent experiments performed in triplicates and normalized to the basal IP1 level of pEGFPN1. Error bars represent S.E.

15 and 30 min significantly triggered receptor internalization (Fig. 7, C and D), thereby demonstrating that the experimental setup is capable of detecting agonist-induced internalization.

Likewise, the real-time internalization assay robustly reported agonist-induced β2AR internalization (Fig. 4, A and B); however, when testing GPRC6A, no increase in internalization was observed upon l-Orn stimulation (Fig. 7E). To detect robust l-Orn-mediated activity in the functional IP-One assay, a 2-h washing protocol was proven necessary (Fig. 2A), but introducing this 2-h washing protocol in the real-time internalization assay also did not cause l-Orn-mediated internalization (Fig. 7F). Importantly, different receptor ligands have been shown capable of inducing distinct trafficking patterns exemplified by the μ-opioid receptor (42). For that reason, other GPRC6A ligands were also tested in the real-time internalization assay. Ca2+ has previously been reported to either directly activate (3, 7) or positively modulate the activity of GPRC6A (2, 4, 5). In the current setup, 10 mM Ca2+ had little effect in the functional IP-One assay but showed a significant potentiating effect when also adding l-Orn (Fig. 7G). Nevertheless, 10 mM Ca2+ was unable to induce GPRC6A internalization although tested both in the presence and absence of l-Orn (Fig. 7H). Although basic l-α-α-amino acids like l-Orn are full agonists at GPRC6A, small and neutral amino acids have been shown to work as partial agonists (5). Accordingly, lower maximum responses were detected upon stimulation of GPRC6A with l-Ala or l-Ile in the IP-One assay (Fig. 7I), but still, neither of these amino acids had any effect on the internalization of GPRC6A (Fig. 7J). Altogether, GPRC6A primarily displays constitutive internalization, and any agonist-induced effects are very minor.

GPRC6A is mainly sorted through the slow recycling pathway

The sorting of internalized receptors can be determined by studying the co-localization of receptors and protein markers for the different endocytic compartments. Rab5 is used as a protein marker for the early endosomes, Rab7 is found in the late endosomes that are part of the receptor degradation pathway, and Rab11 is a marker for the slow recycling pathway that facilitates return of the receptors to the cell surface (24). Antibody feeding was conducted using anti-myc antibodies as described above. Following internalization, cells were fixed and permeabilized, and the different Rab proteins were labeled using primary antibodies. Subsequently, myc-tagged receptors were detected using Alexa Fluor 488-conjugated secondary antibodies, and Rab proteins were detected using Alexa Fluor 568-conjugated secondary antibodies. Two conditions for allowing internalization were tested: 30-min incubation in buffer for constitutive internalization and 15 min of buffer followed by 15 min of 5 mM l-Orn for agonist-induced internalization. Visual inspection of the images suggested prominent overlap of the internalized GPRC6A with Rab5 as well as with Rab11 following both buffer and l-Orn stimulation. A minor overlap between GPRC6A and Rab7 was also observed (Fig. 8, A–C). These findings were substantiated by quantification of the co-localization using van Steensel’s co-localization analysis (43). The Pearson’s cross-correlation factor peaked at 0.44 (Rab5 co-localization), 0.16 (Rab7 co-localization), and 0.58 (Rab11 co-localization) for constitutive internalized GPRC6A, and the decrease in the cross-correlation function (CCF) with increasing x axis offset between the two channels confirmed the specific overlay of the signals. After 15 min of l-Orn stimulation, the CCF of GPRC6A and Rab5 was slightly increased (CCF peak = 0.52) compared with the level of co-localization upon constitutive internalization. On the contrary, the co-localization of GPRC6A and Rab11 was slightly decreased (CCF peak = 0.52) upon l-Orn stimulation. The minor overlap in localization between GPRC6A and Rab7 was unaffected by agonist stimulation (CCF peak = 0.17) (Fig. 8, D–F). Thus, following endocytosis of GPRC6A, the receptor is mainly located in the early endosomes and is subsequently sorted through the slow recycling pathway. A small fraction of the receptors are located in the late endosomes and are thus targeted for degradation.

To further investigate the postendocytic sorting of GPRC6A, co-localization of the receptor and Alexa Fluor 568-conjugated transferrin, a bona fide recycling marker, was studied. Upon binding to transferrin receptors at the cell surface, transferrin undergoes endocytosis and subsequent Rab11-dependent recycling. myc-tagged GPRC6A was labeled using Alexa Fluor 488-conjugated anti-myc antibodies, and Alexa Fluor 568-conjugated transferrin was added. Upon 30-min incubation in buffer, a prominent overlap in localization between internalized GPRC6A and transferrin was observed (Fig. 9A). The specificity of the co-localization was confirmed using van Steensel’s co-localization quantification analysis, which resulted in a CCF peak at 0.72 (Fig. 9B). These results thus further substantiate that GPRC6A is sorted to recycling compartments.

Upon agonist-induced endocytosis, β2AR is likewise sorted through recycling endosomes (25). In the present study, the sorting of β2AR was confirmed by investigating the co-localization with the recycling marker transferrin. FLAG-tagged β2AR was labeled using Alexa Fluor 647-conjugated anti-FLAG antibody, and Alexa Fluor 568-conjugated transferrin was added. Upon stimulation with 10 μM ISO, internalized β2AR co-localized with internalized transferrin as seen from both the microscopy images and van Steensel’s quantification analysis (CCF...
peak at 0.58) (Fig. 9, C and D). Consequently, both transferrin and β₂AR can be used as markers for the recycling pathway. Finally, the localization of GPRC6A was studied upon co-expression with β₂AR. Surface-expressed myc-GPRC6A was labeled using Alexa Fluor 488-conjugated anti-myc antibodies, surface-expressed FLAG-β₂AR was labeled using Alexa Fluor 647-conjugated anti-FLAG antibodies, and Alexa Fluor 568-conjugated transferrin was added. Subsequently, internalization was allowed by incubation in buffer ± 10 μM ISO. In the absence of ISO, only GPRC6A and transferrin were internalized, whereas β₂AR was localized to the cell surface. GPRC6A and transferrin showed prominent overlap in localization (Fig. 9E), consistent with the results shown in Fig. 9A. Also, van Steensel’s analysis showed a prominent peak in CCF (CCF peak = 0.73) for co-localization of internalized GPRC6A and transferrin, whereas very limited co-localization of internalized GPRC6A and β₂AR was observed as expected (CCF peak = 0.15) (Fig. 9F). When stimulating with 10 μM ISO, GPRC6A, β₂AR, and transferrin were all internalized and showed overlap in intracellular localization (Fig. 9E). The specificity of the co-

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A

B

C

D

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F

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localization was verified by use of van Steensel’s quantification analysis, which showed CCF peaks at 0.58 for GPRC6A and transferrin and 0.47 for GPRC6A and β2AR (Fig. 9G). Thus, internalized GPRC6A is co-localized with both transferrin and β2AR, which further confirms that GPRC6A is sorted through recycling.

Discussion

In the present study, we investigated the endocytosis and intracellular sorting of the GPRC6A receptor. Two different methods for measuring receptor internalization were used: the antibody feeding method and a real-time internalization assay. Results from both techniques demonstrated that GPRC6A undergoes robust, constitutive internalization, whereas agonist-induced internalization is very limited. Upon endocytosis, the receptor is found in the early endosomes and in the Rab11-dependent slow recycling pathway, although a smaller fraction of receptors are sorted for degradation. Moreover, internalized GPRC6A was also found to co-localize with the recycling marker transferrin as well as internalized β2AR, which is a well-known recycling receptor.

The antibody feeding internalization assay is by far the most commonly used method for studying receptor endocytosis. It is a powerful technique that allows for visualization of internalization at a single cell level; however, it is also very low-throughput and does not easily enable quantitative dynamic studies. To complement the antibody feeding assay, we used a novel internalization assay based on TR-FRET measurements. The assay allows for detection of receptor internalization in a time-dependent manner and is performed in a 96-well format, thus enabling higher throughput than previously available methods. To verify the real-time internalization technique, two groups tested a range of GPCRs and correlated results from the real-time assay with classical immunocytochemistry experiments (37, 38). Moreover, GLP1R internalization was tested in the presence of a dominant-negative mutant of dynamin, which blocks dynamin-dependent internalization. Co-expressing this dynamin mutant resulted in complete inhibition of the GLP1-induced increase in donor/acceptor ratio, thus verifying that the assay specifically measures receptor internalization (44).

When characterizing the trafficking of GLP1R and β2AR, both receptors showed internalization in response to agonist (37). However, the experimental setup was not applicable for studying constitutively internalizing receptors as the labeling of surface receptors was performed at 37 °C. In the present study, we show that an experimental setup in which labeling is performed at 4 °C successfully detects constitutive internalization of GPRC6A. This is in agreement with Levoye et al. (38) in which constitutive internalization of the CXC chemokine receptor 7 was detected by means of the real-time internalization assay using 4 °C labeling.

It is well established that amino acids are released from cells in culture (45), and these ligands might consequently be triggering the observed internalization of GPRC6A. To minimize effects from omnipresent agonists, we chose to test the rat GPRC6A receptor in the current study as amino acids are less potent at the rat receptor compared with the human receptor. Also, the rat receptor displays higher surface expression compared with the mouse and human orthologs (1, 7, 46), which should result in a higher signal-to-noise response in both the antibody feeding and real-time internalization assays. Accordingly, we were unable to test the human receptor in the current experimental setups due to the lower expression level (data not shown). In future studies it would, however, be of great interest to investigate whether the human GPRC6A receptor displays the same trafficking patterns as the rat ortholog.

Due to the omnipresence of GPRC6A agonists, it was necessary to verify that the receptor internalization was genuinely agonist-independent. Any agonistic effects were effectively precluded by the use of a receptor construct in which the D303A mutation was introduced in the orthosteric binding site to prevent agonist-mediated receptor activation. Conclusively, the D303A mutant successfully demonstrated constitutive internalization in both assays. However, it should be noted that D303A displayed a small decrease in internalization compared with the WT receptor when using the real-time internalization assay, although this most likely reflects the lower expression level of D303A compared with WT GPRC6A. When using the antibody feeding method, no significant difference between D303A and WT was observed, but given that data are depicted as the internalized/surface ratio, the lower surface expression of D303A has already been accounted for in this data analysis. Furthermore, addition of the full agonist L-Orn, the partial agonists L-Ala and L-Ile, the positive modulator Ca2⁺, or the Gq inhibitor FR900359 had no effect on GPRC6A internalization, which further verifies that the internalization is independent of receptor activity. Altogether, these results substantiate that GPRC6A primarily undergoes constitutive internalization, and

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**Figure 7. Agonist-induced internalization of GPRC6A and β2AR.** Confocal microscopy images of HEK293T cells co-transfected with myc-GPRC6A and GqG66D in a 1:1 ratio (A) or transfected with myc-β2AR (C) are shown. Surface receptors were labeled using primary anti-myc antibody for 30 min, and internalization was allowed by 30-min incubation in buffer ± agonist. Specifically, three conditions were tested: 30 min of buffer alone, 15 min of buffer followed by 15 min of agonist, and 30 min of agonist. For A, 5 mM L-Orn was tested, and for C, 9 μM ISO was tested. Insets show high magnification of the regions indicated by white rectangles. Representative images (n = 30) from three independent experiments are shown. Scale bars, 10 μm. Error quantification of A, D, quantification of C. One cell per image was chosen for quantification of surface labeling and internalized labeling, respectively. Data are shown as internalized/surface ratio and normalized to the condition with only buffer. 30 cells from three independent experiments were used for each condition, and the means and standard errors are shown with error bars representing S.E. E and F, real-time internalization measurements of HEK293T cells co-transfected with myc-GPRC6A and GqG66D in a 1:1 ratio. The labeling was performed at 4 °C, buffer ± 5 mM L-Orn was added to allow internalization. F, GPRC6A was tested with or without a 2-h wash prior to labeling. 5 mM L-Orn was added to allow internalization. Data are means of a single representative experiment performed in triplicates with error bars representing S.D. Two additional experiments gave similar results. G and H, the effect of 10 mM Ca2⁺ in the presence or absence of 5 mM L-Orn was tested at SNAP-GPRC6A + GqG66D in the functional IP-One assay (G) and the real-time internalization assay (H). Data are means of four independent experiments performed in triplicates and normalized to the basal IP, level of GPRC6A (G) or shown as area under the curve (AUC) (H). Error bars represent S.E. I and J, the effect of the partial agonists L-Ala and L-Ile was tested at SNAP-GPRC6A + GqG66D in the functional IP-One assay (I) and the real-time internalization assay (J). Data are means of four independent experiments performed in triplicates and normalized to the basal IP, level of GPRC6A (G) or shown as area under the curve (AUC). Error bars represent S.E. Statistical analysis was performed using a one-way analysis of variance followed by Dunnett’s post-test (ns, p > 0.05; *p < 0.05; **p < 0.01; ***p < 0.001).
although agonists are present during cell culturing and assays, they thus do not influence the endocytosis of GPRC6A.

Co-expression of Gq,G66D has proven essential for obtaining functional responses of transiently expressed GPRC6A as it increases the coupling sensitivity of the receptor toward the Gq signaling pathway (4, 5, 26). However, activation of endogenous Gq proteins has also been demonstrated using Chinese hamster ovary (CHO) cells stably expressing mouse GPRC6A, hence verifying the Gq coupling (7). In the present study, we showed that Gq,G66D is not responsible for the constitutive internalization of GPRC6A because co-expression of the receptor with pcDNA3.1 likewise resulted in constitutive internalization. Nevertheless, Gq,G66D does have minor effects on the level of GPRC6A endocytosis. Overexpression of the promiscuous Gq,G66D protein likely affects endogenously expressed GPCRs and in particular any Gq-coupled receptors. An overall increase in sensitivity toward G protein signaling could result in increased activity of the cellular endocytosis machinery, and this might have indirect effects on the level of GPRC6A internalization.

When using the antibody feeding method, a small but significant increase in internalization was detected upon agonist stimulation, whereas no agonist-mediated effects were observed in the real-time internalization assay. Because the real-time assay is being performed in a 96-well format using a high amount of cells and a small volume of buffer, we hypothesize that release of amino acids from cells results in a higher amino}

Figure 8. Co-localization studies of GPRC6A and Rab protein markers. HEK293 cells co-transfected with myc-GPRC6A and Gq,G66D in a 1:1 ratio were tested. Surface receptors were labeled using primary anti-myc antibody for 30 min, and internalization was allowed by 30-min incubation in buffer ± 5 min L-Orn. Specifically, two conditions were tested: 30 min of buffer alone and 15 min of buffer followed by 15 min of L-Orn. Following cell permeabilization, specific anti-Rab antibodies were used for labeling of Rab5 protein marker (A), Rab7 protein marker (B), and Rab11 protein marker (C). Insets show high magnification of the regions indicated by white rectangles. Representative images (n = 30) from three independent experiments are shown. Scale bars, 10 μm. D–F, quantification of co-localization using the JACoP plug-in in ImageJ. van Steensel’s CCF analysis was performed, and the means of CCF are shown as a function of the pixel shift for co-localization with Rab5 (D), Rab7 (E), and Rab11 (F), respectively. Two to four cells per image were chosen for the quantification analysis, and a total of n = 30 images from three independent experiments were used. Error bars represent S.E.
acid concentration surrounding the cells than in the antibody feeding setup. Consequently, addition of L-Orn might not induce further internalization in the real-time assay.

Applying agonist in the antibody feeding setup resulted in an initial increase in endocytosis after 15 min of L-Orn stimulation; however, internalization was yet again lowered after 30 min of
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L-Orn stimulation. The number of internalized receptors is evidently dependent on the rate of internalization but is also influenced by the rate of recycling and degradation. Assumedly, the level of constitutively internalized GPRC6A receptors represents an equilibrium between internalization and recycling/degradation. L-Orn stimulation seems to shift this equilibrium by slightly increasing the rate of internalization, which is observed as the small increase in internalized receptors after 15 min of stimulation. Results from the co-localization studies showed that internalized GPRC6A was found mainly in the early endosomes and the Rab11-dependent slow recycling pathway and less so in the Rab7-dependent pathway; however, L-Orn stimulation redirected a small amount of receptors away from the slow recycling pathway, whereas the sorting to the degradation pathway was unaffected. It could thus be suggested that L-Orn redirects a small portion of the receptors to the fast recycling pathway, which would marginally increase the rate of recycling. An L-Orn-mediated increase in recycling rate would explain the decrease in internalization from 15 to 30 min of L-Orn as the latter could represent the new equilibrium in which the small L-Orn-induced increase in internalization is counteracted by a subsequent small increase in recycling rate.

Cells in culture expressing GPRC6A are constantly exposed to receptor agonists as amino acids and cations are vital components of cell growth media. For the majority of GPCRs, a constant exposure to agonists would result in receptor desensitization and subsequent receptor endocytosis to protect the cells from overstimulation (22). Previously, we have shown that a washing step is essential for obtaining functional responses of mouse GPRC6A in transient expression systems (4). This washing step was also shown to be important for rat GPRC6A in the present study. We hypothesized that the washing protocol is responsible for removing GPRC6A agonists prior to the assay, thereby preventing receptor desensitization and endocytosis and consequently increasing the amount of receptors in the cell membrane. Results from this study, however, demonstrate that including a washing protocol did not decrease the level of internalization or increase the surface expression of GPRC6A. The observed effect of washing in functional assays is consequently not caused by prevention of endocytosis prior to the assay. Nevertheless, washing away agonists might still prevent desensitization, thereby allowing detection of receptor activation. This could suggest that agonist-mediated activation of GPRC6A is regulated by desensitization of the receptor alone and not receptor endocytosis, which is otherwise the case for most GPCRs.

GPRC6A belongs to the class C receptor subfamily, which is a group of nutrient-sensing receptors activated by various amino acids and cations (47). Accordingly, most of these receptors are under prolonged or constant exposure to agonists. Although trafficking has not been studied in detail for all the class C receptors, it seems that several of them are not subjected to the classical regulatory mechanisms. Although agonist-mediated endocytosis has been observed for several of the mGlURs, mGlur1 and mGlur5 additionally demonstrate constitutive internalization (48, 49). CaSR is the closest related mammalian receptor of GPRC6A and has proven highly resistant to desensitization, which most likely reflects a persistent exposure to the endogenous agonist Ca2+ (50, 51). Constitutive internalization of CaSR has been observed in some studies (52, 53), and moreover, a distinct regulatory mechanism for CaSR has been reported in which agonist stimulation triggers further insertion of receptors in the cell membrane from intracellular stores, thereby increasing the amount of receptors available for activation. This mechanism has been named agonist-driven insertion (54). Finally, the γ-aminobutyric acid B (GABA_B) receptors do not internalize in response to agonist; however, they do exhibit constitutive internalization and subsequent recycling (55–57) in line with our current observations for the GPRC6A receptor. Such distinct regulatory mechanisms might have evolved for class C GPCRs to ensure a functional receptor pool in the cell membrane despite the constant presence of agonists. Accordingly, the constitutive internalization and recycling of GPRC6A enable a continual level of receptors at the cell surface and a pool of internalized receptors that are available for recycling. It is possible that other regulatory mechanisms are responsible for increasing the rate of recycling, hence increasing the surface expression of GPRC6A whenever necessary, as has been observed for CaSR.

In conclusion, we have shown that the GPRC6A receptor is internalized and recycled constitutively, and these events consequently do not seem to directly regulate the agonist-mediated receptor response. They do, however, affect the level of receptor activity by ensuring a persistent amount of receptors at the cell surface. These findings are highly important in our understanding of GPRC6A function and regulation; however, much is yet to be elucidated. Future studies could clarify whether receptor phosphorylation and β-arrestin recruitment are involved in the regulation and to what extent GPRC6A function is controlled by receptor desensitization.

Experimental procedures

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Dulbecco’s modified Eagle’s medium (DMEM), penicillin/streptomycin mixture, dialyzed FBS, Opti-MEM, Dulbecco’s phosphate-buffered saline (DPBS), Hanks’ balanced...
salt solution (HBSS) (catalog number 14175-129), and Lipofectamine 2000 were all purchased from Thermo Fisher Scientific (Waltham, MA). The Gq inhibitor FR900359 was purchased from Prof. Evi Kostenis (University of Bonn, Bonn, Germany).

**Constructs**

The previously described N-terminally c-myc-tagged rat GPRC6A (rGPRC6A) construct in which the receptor has been inserted in the pEGFPN1 vector downstream of an mGluR5 signal peptide (S) was used in the present study. The myc-rGPRC6A-D303A mutant was generated by Genscript (Piscataway, N) using the myc-tagged rGPRC6A construct as template. myc-tagged β2AR was generated by replacing the GPRC6A sequence with the mouse β2AR sequence in the myc-GPRC6A-pEGFPN1 construct by using the MluI and NotI restriction sites. HA-rGPRC6A WT was generated by replacing the myc tag in the myc-rGPRC6A-pEGFPN1 construct with an HA tag. SNAP-rGPRC6A WT and SNAP-rGPRC6A-D303A were generated by replacing the mGluR5 signal peptide and the myc tag in the myc-rGPRC6A-pEGFPN1 constructs with a T-cell CD8A signal peptide and SNAP tag from a SNAP-tagged GLP1R construct (37) by using Xhol and Mulu restriction sites. SNAP-GLP1R and SNAP-β2AR were kindly provided by Dr. Maria Waldhoer (Novo Nordisk A/S), whereas FLAG-β2AR was kindly provided by Dr. Mark von Zastrow.

**Transient transfection**

HEK293T cells were cultured in DMEM supplemented with 1% penicillin/streptomycin and 10% dialyzed FBS at 37 °C and 5% CO2. 48 h before assay, transient transfection in HEK293T cells was performed in white, opaque 96-well culture plates (PerkinElmer Life Sciences) for ELISA; clear tissue culture treated 96-well plates (Corning, Corning, NY) for the functional IP-One assay; and white, opaque 96-well culture plates (Corning) for the real-time internalization assay. Prior to transfection, all plates were coated using poly-d-lysine. For each well, 0.25 μg of DNA (for testing GPRC6A and GqG66D in a 1:1 ratio) or 0.125 μg of DNA (for testing β2AR) was diluted in 25 μl of Opti-MEM. Likewise, 0.25 μl/well Lipofectamine 2000 was diluted in 25 μl of Opti-MEM and incubated at room temperature for 5 min. Subsequently, DNA and Lipofectamine 2000 dilutions were mixed and further incubated for 20 min at room temperature before addition to the plate. HEK293T cells were prepared in complete growth medium at a density of 170,000 cells/ml of which 100 μl was added to each well (17,000 cells/well). Plates were then incubated for 48 h at 37 °C and 5% CO2. For the antibody feeding experiments, 200,000 HEK293 cells/well were seeded in complete growth medium on poly-d-lysine-coated 16-mm-diameter coverslips (Menzel-Gläser, VWR, Radnor, PA) in clear 12-well culture plates (Sigma-Aldrich). Following 24 h of incubation at 37 °C and 5% CO2, transient transfection was performed using 0.5 μg/well DNA (for testing GPRC6A and GqG66D in a 1:1 ratio or for testing GPRC6A, β2AR, and GqG66D in a 1:1:1 ratio) or 0.25 μg/well DNA (for testing β2AR) and 1.5 μl/well Lipofectamine 2000. DNA and Lipofectamine 2000 were each diluted in 50 μl/well Opti-MEM and incubated for 5 min at room temperature. Subsequently, the dilutions were mixed and incubated for 20 min at room temperature before addition to the plate. 900 μl/well Opti-MEM was also added. Plates were incubated at 37 °C and 5% CO2 for 4–6 h after which the transfection mixture was replaced with complete growth medium. Plates were then incubated for 48 h at 37 °C and 5% CO2.

**Internalization and sorting of the GPRC6A receptor**

The expression level of myc-tagged GPRC6A was determined using ELISA as described previously (21). In brief, cells were fixed in 50 μl/well 4% paraformaldehyde (PFA) in DPBS for 5 min at room temperature. Wells for determination of total expression were incubated with 0.1% Triton-X in DPBS to allow cell permeabilization, and all wells were subsequently incubated with blocking solution (double distilled H2O with 3% skim milk, 1 mM Ca2+, 50 mM Trizma hydrochloride (Tris base) solution, pH 7.4) for 30 min. Primary antibody incubation was performed using a mouse anti-myc antibody (Thermo Fisher Scientific) followed by secondary antibody incubation using horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare). Detection was performed by addition of 80 μl/well DPBS and 10 μl/well SuperSignal ELISA Femto Substrate (Thermo Fisher Scientific). Immediately afterward, chemiluminescence was measured on an EnSpire plate reader (PerkinElmer Life Sciences).

**Functional IP-One assay**

Functional responses of GPRC6A were measured using a TR-FRET-based assay as described previously (21). In brief, cells were washed 2 × 2 h in washing buffer (HBSS, 20 mM HEPES, 0.5 mM CaCl2, 0.5 mM MgCl2, 1 mg/ml bovine serum albumin, pH 7.4) followed by one wash in assay buffer (HBSS, 20 mM HEPES, 1 mM CaCl2, 1 mM MgCl2, pH 7.4). Ligands were prepared in ligand buffer (assy buffer supplemented with 40 mM LiCl), and 50 μl/well ligand solution was added. Cells were stimulated for 1 h at 37 °C after which wells were washed once with assay buffer. When testing the Gq-specific inhibitor FR900359, cells were preincubated in 1 μM FR900359 for 1 h at 37 °C, and subsequently, cells were stimulated with 1 μM FR900359 agonist for 1 h at 37 °C. Cell lysis was performed using 30 μl/well IP-One Conjugate and Lysis Buffer (Cisbio Bioassays, Codolet, France), and 10 μl/well cell lysate was subsequently transferred from the 96-well culture plate to a white 384-well OptiPlate (PerkinElmer Life Sciences). 10 μl of detection solution (2.5% of anti-IP1 antibody-terbium cryptate conjugate + 2.5% IP1-d2 conjugate (Cisbio Bioassays) in assay buffer) was added to each well, and the plate was incubated for 1 h at room temperature in the dark before being measured on an EnVision plate reader (PerkinElmer Life Sciences). Upon excitation at 340 nm, emissions at 615 and 665 nm were measured, and the FRET ratio (665 nm/615 nm) was converted to IP1 concentrations using a standard curve generated from an IP1 calibrator provided by the manufacturer (Cisbio Bioassays).

**Antibody feeding internalization assay**

Primary antibody incubation was performed using a mouse anti-myc antibody (Sigma-Aldrich) diluted 1:2000 in assay buffer (HBSS, 20 mM HEPES, 0.5 mM CaCl2, 0.5 mM MgCl2, pH 7.4). 1 h at 37 °C after which wells were washed once with assay buffer. When testing the Gq-specific inhibitor FR900359, cells were preincubated in 1 μM FR900359 for 1 h at 37 °C, and subsequently, cells were stimulated with 1 μM FR900359 agonist for 1 h at 37 °C. Cell lysis was performed using 30 μl/well IP-One Conjugate and Lysis Buffer (Cisbio Bioassays, Codolet, France), and 10 μl/well cell lysate was subsequently transferred from the 96-well culture plate to a white 384-well OptiPlate (PerkinElmer Life Sciences). 10 μl of detection solution (2.5% of anti-IP1 antibody-terbium cryptate conjugate + 2.5% IP1-d2 conjugate (Cisbio Bioassays) in assay buffer) was added to each well, and the plate was incubated for 1 h at room temperature in the dark before being measured on an EnVision plate reader (PerkinElmer Life Sciences). Upon excitation at 340 nm, emissions at 615 and 665 nm were measured, and the FRET ratio (665 nm/615 nm) was converted to IP1 concentrations using a standard curve generated from an IP1 calibrator provided by the manufacturer (Cisbio Bioassays).

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The expression level of myc-tagged GPRC6A was determined using ELISA as described previously (21). In brief, cells were fixed using 50 μl/well 4% paraformaldehyde (PFA) in DPBS for 5 min at room temperature. Wells for determination of total expression were incubated with 0.1% Triton-X in DPBS to allow cell permeabilization, and all wells were subsequently incubated with blocking solution (double distilled H2O with 3% skim milk, 1 mM Ca2+, 50 mM Trizma hydrochloride (Tris base) solution, pH 7.4) for 30 min. Primary antibody incubation was performed using a mouse anti-myc antibody (Thermo Fisher Scientific) followed by secondary antibody incubation using horseradish peroxidase-conjugated anti-mouse antibody (GE Healthcare). Detection was performed by addition of 80 μl/well DPBS and 10 μl/well SuperSignal ELISA Femto Substrate (Thermo Fisher Scientific). Immediately afterward, chemiluminescence was measured on an EnSpire plate reader (PerkinElmer Life Sciences).
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7.4) for 30 min at 37 °C for labeling of surface-expressed receptors. Afterward, cells were washed once in assay buffer and then incubated 30 min at 37 °C in buffer with or without agonist to allow internalization. Three different conditions were tested: 30 min in buffer, 15 min of buffer followed by 15 min of agonist, and 30 min of agonist. Subsequently, cells were fixed by addition of 4% PFA in DPBS for 15 min at room temperature. The coverslips were washed three times in DPBS and incubated with blocking buffer (5% goat serum in DPBS) for 20 min at room temperature. Labeled surface receptors were detected by incubation with goat anti-mouse Alexa Fluor 568-conjugated antibody (Thermo Fisher Scientific) diluted 1:100 in blocking buffer for 1 h at 37 °C. Cells were washed three times with DPBS and permeabilized by using 0.2% saponin in blocking buffer for 20 min at room temperature. Internalized receptors were detected by incubation with goat anti-rabbit Alexa Fluor 488-conjugated antibody (Thermo Fisher Scientific) diluted 1:500 in blocking buffer for 40 min at 37 °C, which was followed by three washing steps with DPBS before cells were mounted in ProLong Gold antifade mountant (Molecular Probes, Thermo Fisher Scientific). When testing HA-tagged GPRC6A, the protocol was as stated above; however, labeling was performed using rat anti-HA antibody (Roche Diagnostics GmbH) diluted 1:1000 in assay buffer for 30 min at 4 °C. Surface receptors were detected using goat anti-rat Alexa Fluor 568-conjugated antibody (Thermo Fisher Scientific) diluted 1:100 in blocking buffer, and internalized receptors were detected using goat anti-rat Alexa Fluor 488-conjugated antibody (Thermo Fisher Scientific) diluted 1:500 in blocking buffer. When testing SNAP-GPRC6A, surface receptor labeling was performed using an Alexa Fluor 488-conjugated SNAP substrate (New England Biolabs, Ipswich, MA) diluted 1:200 in Opti-MEM supplemented with 0.5% bovine serum albumin. Labeling was performed at 4 °C for 30 min. Subsequently, cells were washed once in assay buffer and then incubated for 30 min at 37 °C in assay buffer to allow internalization. Cells were fixed in 4% PFA, washed three times in DPBS, and mounted in ProLong Gold antifade mountant. In all experiments, cells were visualized by confocal microscopy. Images were chosen and analyzed in a blinded fashion.

Co-localization with Rab protein markers

Labeling and internalization of myc-tagged surface receptors and fixation of cells were performed as described above for the antibody feeding internalization assay. Two different internalization conditions were tested: 30 min in buffer and 15 min of buffer followed by 15 min of agonist. Following fixation, cells were washed three times in DPBS and permeabilized by incubation in 0.2% saponin in blocking buffer for 20 min at room temperature. Rab protein markers were labeled by incubation for 1 h at 37 °C with rabbit anti-Rab antibodies (for Rab5, Rab7, and Rab11, respectively) (Cell Signaling Technology, Danvers, MA) diluted 1:500 in blocking buffer. Cells were subsequently washed three times in DPBS prior to incubation with secondary antibodies. Goat anti-mouse Alexa Fluor 488-conjugated antibody diluted 1:500 in blocking buffer was used for detection of myc-tagged receptors, and goat anti-rabbit Alexa Fluor 568-conjugated antibody (Thermo Fisher Scientific) diluted 1:500 in blocking buffer was used for detection of Rab proteins. After 40 min of incubation at 37 °C, cells were washed three times in DPBS and mounted using ProLong Gold antifade mountant. Cells were visualized by confocal microscopy. Images were chosen and analyzed in a blinded fashion.

Co-localization with transferrin

Surface receptors were labeled using a mouse anti-myc Alexa Fluor 488-conjugated antibody (for myc-GPRC6A) (Thermo Fisher Scientific) diluted 1:500 in assay buffer (HBSS, 20 mM HEPES, 0.5 mM CaCl₂, 0.5 mM MgCl₂, pH 7.4) and a mouse anti-FLAG Alexa Fluor 567-conjugated antibody (for FLAG-β₂AR) (Sigma-Aldrich) diluted 1:500 in assay buffer. Labeling was performed for 30 min at 37 °C in the presence of 0.1 mg/liter FeCl₃ and Alexa Fluor 568-conjugated transferrin (Molecular Probes, Thermo Fisher Scientific) diluted 1:200. Subsequently, cells were washed once in assay buffer and incubated for 30 min at 37 °C in assay buffer ± 10 μM ISO. 0.1 mg/liter FeCl₃ was present during this stimulation time. Afterward, cells were fixed using 4% PFA, washed three times in DPBS, and mounted using ProLong Gold antifade mountant. Cells were visualized by confocal microscopy. Images were chosen and analyzed in a blinded fashion.

Confocal microscopy

Imaging was performed using a Zeiss LSM 510 inverted confocal laser-scanning microscope with an oil immersion numerical aperture 63 × 1.4 objective (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Alexa Fluor 568 was excited using a 543-nm helium-neon laser, and emission was measured using a 560-nm long-pass filter. Alexa Fluor 488 was excited by a 488-nm laser line from an argon-krypton laser, and emitted light was filtered using a 505–550-nm band-pass filter. Alexa Fluor 647 was exited by a 633-nm helium-neon laser, and emission was measured using a 650-nm long-pass filter. Channels were imaged separately, and resulting images were merged using ImageJ software (Wayne Rasband, National Institutes of Health).

Real-time internalization assay

Labeling of surface receptors was performed by incubating cells for 1 h at 4 °C (or 37 °C) with 50 μM/well 100 nM Tag-lite SNAP Lumi4-Tb (Cisbio Bioassays) diluted 1:1000 in Opti-MEM. Ligands were prepared in 2× final concentration in assay buffer (HBSS, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4), and 100 μM fluorescein was prepared at a dilution of 1:2500 in assay buffer. The ligand dilutions and the fluorescein dilution were then preheated to 37 °C. After receptor labeling, cells were washed twice in cold assay buffer followed by addition of 50 μM/well fluorescein as well as 50 μM/well ligand solution. When testing the G₄i inhibitor FR900359, 1 μM FR900359 (final concentration) was present both during the labeling and during the ligand stimulation. 100 μM/well 37 °C assay buffer was added for measurements of donor signal alone. Immediately afterward, the plate was read using an EnVision plate reader, which was kept under 37 °C temperature control. Every 6th min, emissions at 520 and 615 nm were measured after excitation at 340 nm, and internalization was depicted as the (donor emission, 615 nm)/(acceptor emission, 520 nm) ratio.
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Data analysis

Data were analyzed using GraphPad Prism v.6 (GraphPad Software, San Diego, CA) and Image v.1.47. All statistical analyses were performed as indicated in the figure legends with statistical significance determined at the following levels: not significant (ns), \( p > 0.05 \); *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \). Quantification of microscopy images was performed on single cells to avoid background noise. For all studies, three independent experiments were quantified by using 10 images from each experiment. Images from the antibody feeding internalization assays were quantified by measuring the intensity from each channel (surface labeling and internalized labeling, respectively) on a single cell with threshold pixel intensities set to avoid nonspecific signal. The surface/internalized intensity ratio was calculated for each image and depicted. Images from the co-localization experiments were quantified using the JACoP plug-in for ImageJ in which van Steensel’s CCF analysis was performed (43). Threshold pixel intensities were set to avoid nonspecific staining.

Author contributions—S. E. J. designed, performed, and analyzed all the experiments and wrote the manuscript. I. A.-J. and A. M. J. were involved in designing the antibody feeding experiments and assisted with data analysis. U. G. and K. L. M. supervised the project, were involved in the experimental design, performed data interpretation, and wrote the paper. H. B.-O. conceived the idea for the project, supervised the project, performed data interpretation, and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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