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Internalized Receptor for Glucose-dependent Insulinotropic Peptide stimulates adenylyl cyclase on early endosomes

Sadek Ismail, Marie-Julie Gherardi, Alexander Froese, Madjid Zanoun, Véronique Gigoux, Pascal Clerc, Frederique Gaits-Iacovoni, Jan Steyaert, Viacheslav O. Nikolaev, Daniel Fourmy.

1. Introduction

Seven-transmembrane receptors, also termed G-protein-coupled receptors (7 TMRs or GPCRs), form the largest class of cell surface membrane receptors, involving several hundred members in the human genome. Biological effects triggered by GPCRs result from activation of both G-protein-dependent and G-protein-independent intracellular signaling pathways [1–3]. Until very recently, production of diffusible second messengers was thought to originate exclusively from the plasma membrane and internalized GPCRs were considered silent. Here, we demonstrated that, once internalized and located in the membrane of early endosomes, glucose-dependent Insulinotropic receptor (GIPR) continues to trigger production of cAMP and PKA activation. Direct evidence is based on identification of the active form of Gαs in early endosomes containing GIPR using a genetically encoded GFP tagged nanobody, and on detection of a distinct FRET signal accounting for cAMP production at the surface of endosomes containing GIP, compared to endosomes without GIP. Furthermore, decrease of the sustained phase of cAMP production and PKA activation kinetics as well as reversibility of cAMP production and PKA activity following GIP washout in cells treated with a pharmacological inhibitor of GIPR internalization, and continuous increase of cAMP level over time in the presence of dominant-negative Rab7, which causes accumulation of early endosomes in cells, were noticed. Hence the GIPR joins the few GPCRs which signal through G-proteins both at plasma membrane and on endosomes.

Abbreviations: GIPR, glucose-dependent Insulinotropic Peptide Receptor; cAMP, 3',5'-cyclic adenosine monophosphate; BRET, Bioluminescence Resonance Energy Transfer; FRET, Fluorescence Resonance Energy Transfer.

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challenged and refuted. Evidence of endosomal G-protein signaling of GPCRs was obtained with the parathyroid hormone receptor (PTHr) which induces sustained or persistent production of cAMP in response to PTH stimulation [5]. In kinetic studies, the sustained phase of cAMP production persisted after agonist removal. Concomitantly, sustained production of cAMP, ascribed to internalized Thyroid Stimulating Hormone Receptor (TSHR), was documented in native thyroid follicles isolated from transgenic mice expressing a FRET sensor of cAMP [6]. Dopamine 1 receptor and vasopressin receptors were subsequently shown to trigger cAMP production from endosomes [7,8]. Among the different experimental arguments provided in support of the concept of endosomal cAMP production, chemical and genetically encoded inhibitors of GPCR internalization such as dynasore and dynamin dominant-negative mutants, were shown to affect the sustained phase of cAMP production [5,6]. Additional and more direct evidences were provided in studies with the β2-adrenergic receptor in which conformational biosensors, consisting of nanobodies, enabled identification, by confocal microscopy, of the active form of the β2-receptor, as well as the active Gαs subunit in early endosomes of living cells [9]. Interestingly, in agreement with the concept of compartmentalized cAMP signaling originally proposed in a study on cardiac myocytes, accumulating data support that cAMP produced from endosomes by activated GPCRs causes qualitatively different physiological effects compared to cAMP generated from the plasma membrane [5,6,10–12].

However, despite reasonably convincing evidence that internalized GPCRs continue to trigger G-protein dependent signals, this view has been refuted and still remains a subject of controversy [13,14]. Furthermore, G-protein-dependent production of diffusible second messengers by GPCRs in endosomes was only recently reported for a very restricted number of GPCRs (recently reviewed in [15]). Additionally, direct detection of cAMP production on endosomes resulting from activation of an internalized GPCR was not provided so far. This prompted us to investigate if the Glucose-dependent Insulinotropic Receptor (GIPR) which is a physiologically and pharmacologically important receptor regulating glucose and lipid homeostasis [16] and which is a universal GPCR overexpressed in neuroendocrine tumors [17], would also be endowed of capability to signal from early endosomes. GIPR which belongs to the subfamily-2 of GPCR, triggers Gs-mediated cAMP production and subsequent signaling cascades [16]. We have shown that GIPR undergoes rapid abundant internalization following stimulation by GIP and that internalized GIPR is mainly sorted to the lysosomal degradation pathway [18]. Interestingly, in contrast to most GPCRs, including the closely structurally and functionally related GLP1 receptor, GIPR is internalized independently of β-arrestin recruitment [18,19].

In the current study, by using an integrative approach comprised of live cell confocal microscopy, pharmacological and genetically encoded tools, BRET and FRET measurement of cAMP and PKA activity in whole cells, immune-detection of activated form ofGs and FRET cAMP measurements at the endosome surface (see Fig. 1), we unequivocally demonstrate that internalized GIPR remains active and triggers cAMP production at endosomes. Thus, GIPR joins the group of GPCRs which are activated at the plasma membrane and once, activated and internalized continue to stimulate production of their cognate diffusible second messenger.

### 2. Materials and methods

#### 2.1. Materials

Fragment 1–30 of human GIP (termed GIP) and DY647 labeled-GIP (termed DY647-GIP) were obtained as previously described [20]. The fluorescent probe was highly specific of GIPR (less than 5% nonspecific labeling in the presence of 100-fold excess of unlabeled peptide). Radio-labeled GIP was obtained by radioiodination of Phe1-GIP(1–30) with 125I-Na (Perkin Elmer, France) in the presence of chloramine T and was HPLC purified on a C-18 column. 125I-Phe1-GIP bound to a single class of GIPR binding sites from HEK 293T or Flip-InTM-GIPR-293 cells with a dissociation constant, Kd of 75.7 ± 8.4 nM. Sequence encoding short variant of the human GIPR was derived from a plasmid kindly given by Professor Bernard Thorens (Lausanne, Switzerland) [21]. Chemicals were from the following sources: Dynogo-4a from Abcam (Cambridge, UK), Forskolin from Sigma-Aldrich (St. Quentin Fallavier, France).

Plasmids encodingDsRed tagged Rab5 (termedDsRed-Rab5), DsRed tagged Rab7-DN (termedDsRed-Rab7-DN), GFP tagged EEA1 (termed GFP-EEA1) were supplied by Addgene (www.addgene.org). Plasmid for BRET measurements of cAMP production, namely RLuc-Epac1-citrine was kindly provided by Professor Marc Caron.

The cytosolic cAMP sensor Epac1-camps sequence [22] was used as a backbone to construct the EYFP-Epac1-ECFP-FYVE sensor targeted to early endosomes. After removal of stop codon, the FYVE sequence was amplified by PCR using the primers AAG GAT CCA TGG TGG ATT TCT TCT GCT GGC AAT CTA GTC AAC GGTCCCTTGGTAATCCCTTGGAGG. The fluorescent probe was highly specific of GIPR (less than 5% nonspecific labeling in the presence of 100-fold excess of unlabelled peptide).

In vivo labeling was performed using the Flip-In™ system (Invitrogen). The cell lines were maintained in Dulbecco’s Modified Eagle’s medium supplemented with 10% of fetal bovine serum (FBS), in a humidified atmosphere at 95% air and 5% CO2. Transfections for BRET experiments were performed using polyethyleneimine (PEI) transfection reagent (1 mg/mL, pH 7.4) (Polypeus, Illkirch, France). Plasmids were diluted in DMEM without FBS (ratio DNA (μg)/PEI (μL) 1:3). The mixture was mixed for 15 s on a vortex, incubated for 15 min at room temperature and then deposited on the cells. For confocal experiments, transfections were performed using Lipofectamine 2000 (Invitrogen Life Technologies) following provider’s instructions (ratio DNA (μg)/LFP2000 (μL) 1:2).

### 2.3. BRET assay of cAMP production

Flip-InTMGIPR-293 cells were plated onto 10-cm culture dishes and overnight grown afterward they were co-transfected with a total amount of 5 μg DNA plasmid comprising 4 μg of cAMP BRET biosensor RLuc-Epac-Citrine completed with 1 μg of non-coding plasmid. 24 h after transfection, cells were plated in 96-well clear bottom plates (Corning) at a density of 100,000 cells per well in phenol red free DMEM 2% FBS. After an overnight incubation, the medium was removed and replaced by calcium and magnesium free PBS. BRET assay was initiated by adding 10 μl of coelenterazine h to the wells (final concentration 5 μM). After 10 min of incubation with coelenterazine h, stimulant of cAMP production, namely GIP or Forskolin was injected. Live-time measurements were recorded at 37°C every 5 s for 60 min. Luminescence and fluorescence readings were performed on a Mithras LB940 instru-
ment (Berthold France, Thoiry, France) that allows the sequential integration of signals at 465–505 nm and 515–555 nm windows. MicroWin 2000 software was used for calculation.

2.4. Live cell confocal fluorescence imaging

After an overnight transfection with 2 µg/well of pcDNA5/FRT containing cDNAs of interest, cells were transferred onto poly-L-lysine (Sigma-Aldrich) coated 4-well Lab-Tek chambered coverglass (Nunc). 24 h later, the culture medium was replaced by phenol red free medium (DMEM1X, 4.5 g/L D-glucose, L-Glutamine, 25 mM HEPES, without sodium pyruvate, Gibco Lifetechnologies). Cells were stimulated with appropriate ligand and were imaged at 37 °C with a confocal Zeiss Laser Scanning Microscope LSM-780 equipped with 63×/1.4 NA immersion objective, a GaAsP detector with quantum yield of 45% and a parallel spectral detection attending 32 channels simultaneously in lambda (λ) mode. For multi-color imaging, the fluorophores were excited by the corresponding lasers and the fluorescence signals were collected in the corresponding emission spectra simultaneously. Images were processed using ImageJ software.

2.5. Confocal microscopy image quantification of colocalisation

For co-localization determination both visual inspection and JACOP Image J plugin (Mander’s coefficient) were used. Briefly, for evaluating the co-localization of DY647-GIP with DsRed-Rab5 or GFP-EEA1 (3), Dyngo4-a (4), an inhibitor of dynamin, served to inhibit GIPR internalization whereas DsRed-DN-Rab7 (5) caused accumulation of early endosomes. Activity of GIPR in early endosomes was detected using a nanobody specific of active form of Goα subunit (6) and an endosomal FRET sensor genetically targeted to early endosomes thanks to FYVE sequence recognizing PI(3)P (7). Activity of PKA was detected using AKAR3 FRET sensor (8).

2.6. FRET assays of cAMP production and PKA activity

cAMP production was determined by FRET on Flp-InTMGIPR-293 cells transfected with cytosolic cAMP sensor Epac1-camps plasmid [22]. FRET was monitored as the ratio between emission
at 535 ± 20 nm (YFP) and emission at 480 ± 615 nm (CFP), upon excitation at 436 ± 10 nm using MetaFluor 5.0 software (Molecular Devices). The imaging data were analyzed utilizing MetaMorph 5.0 (Molecular Devices) and Prism (GraphPad Software) software, by correcting for spillover of CFP into the 535-nm channel and direct YFP-excitation, to give corrected YFP/CFP ratio data. Images were acquired every 5 s, with 5-ms illumination time, which resulted in negligible photobleaching for over 30-min observation. To study GIP-induced changes and reversibility in cAMP, cells were continuously superfused with phenol red-free medium containing 1% BSA or the same plus GIP and/or internalization inhibitor, Dyno-4a, with a custom apparatus. All experiments were performed at 25 °C.

FRET assay of cAMP from endosomes was carried out on a confocal Zeiss Laser Scanning Microscope LSM-780. FRET was monitored as the ratio between emission at 439 ± 23 nm (YFP) and emission at 482 ± 18 nm (CFP) upon excitation at 458 nm. The DY647-GIP emission at 485 ± 40 nm was detected in sequential mode upon excitation at 633 nm. By using this setup and under our experiment conditions, we verified experimentally that GIP-DY647 fluorochrome did not influence signal of cAMP FRET biosensor (not shown). Images were analyzed using ImageJ. Threshold limited endosomes were outlined using “analyze particle” tool. Mean FRET value for each endosome is calculated on the FRET resulting image. Endosome state, namely internalized (containing DY647-GIP) or pre-existing (free of ligand), was visually determined on the merged image.

PKA activity was detected using FRET-based A Kinase Activity Reporter (AKAR3) sensor previously introduced [23]. This sensor was composed of a pair of fluorescent moieties (eCFP and eYFP) sandwiching a molecular probe composed of a surrogate substrate for PKA and a phosphoamino acid binding domain (FHA). Following phosphorylation of the substrate by PKA, the FHA domain binds the phosphorylated amino acid causing a conformational change which results in FRET signal increase. FRET was monitored and analyzed as described for cAMP measurements.

2.7. Receptor binding assays

Flp-In™GIPR-293 cells were grown onto 10-cm culture dishes for 24 h and then transferred to 24-well plates. Approximately 24 h later, binding assays were performed using 125I-Phe1-GIP according to the protocol previously described in detail [25]. Non-specific binding corresponded to residual binding of 125I-Phe1-GIP in the presence of 1 μM GIP. IC50 (concentration inhibiting half of specific binding) was calculated using the non-linear curve fitting software GraphPad Prism (San Diego, CA).

2.8. Statistics

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analyses of data using Student’s t-test were performed with GraphPad Prism software version 6.0. Significance degrees were given as following: *p < 0.05; **p < 0.01; ***p < 0.001.

3. Results

3.1. Inhibition of GIPR sorting to early endosomes affected the sustained phase of cAMP production

We investigated production of cAMP from early endosomes in HEK cells which has been proven to be an excellent and relevant cell model for the study of GPCR internalization and related signaling. HEK 293 cells stably expressing GIPR, (termed Flp-In™ HEK-GIPR), bound GIP with a nanomolar affinity and dose-dependently responded to GIP by stimulating production of cAMP with an EC50 of 1 ± 0.2 nM [18]. We traced GIPR at the cell surface or interior by using fluorescently tagged GIP (termed DY647-GIP) which bound to GIPR and stimulated cAMP production identically to unmodified GIP and, moreover, co-localized with GIPR for up to 2 h during post-endocytic sorting to lysosomes [18]. As shown on Fig. 2A, confocal microscopy observations of Flp-In™ HEK-GIPR cells revealed intense homogenous labeling of the plasma membrane immediately after addition of DY647-GIP. Fluorescence was then rapidly relocated in membrane clusters and rapidly entered into the cells as punctuate vesicles which moved over the incubation time. Confocal microscopy imaging of cells expressing tagged functional proteins of early endosomes, namely DsRed-Rab5 or GFP-EEA1, indicated that chronic stimulation of GIPR resulted in abundant localization of DY647-GIP-bound GIPR in vesicles positive for the markers of early endosomes (Fig. 2B–D).

In order to determine whether GIPR can signal from endosomes to produce cAMP, we first sought to assess the influence of internalization blockade on the kinetic and level of GIP-induced cAMP response measured in real-time by a cytosolic BRET biosensor. Internalization of GIPR occurs through clathrin-coated pits, with an involvement of dynamin that enables detachment of endocytic vesicles from the plasma membrane and formation of early endosomes containing GIPR [18] (Fig. 1). In light of previous data showing that the chemical dynamin inhibitor Dynasore was not fully effective to arrest GIPR internalization, we tested Dyno-4a which was reported to be ~6-fold more potent than dynasore to inhibit dynamin in intact cells [26].

We therefore determined if Dyno-4a affected kinetic and real-time levels of GIP-stimulated cAMP formation. As shown on Fig. 3, cAMP stimulated by 10 nM GIP rapidly increased and remained sustained at its maximum level over time. In contrast, cAMP level continuously declined in the presence of Dyno-4a to a value of ~27% of the maximum value at 60 min of stimulation, suggesting the existence of a relationship between cAMP production and GIPR internalization. Profile of cAMP production stimulated by 100nM GIP was slightly different. It showed a rapid increase followed by a slight drop, likely due to strong desensitization of the initial cAMP response at this GIP concentration until 10 min of stimulation [18]. Then, cAMP level remained constant to ~70% of the maximum value.

We also assessed that sustained phase of cAMP production truly result from internalized GIPR by studying reversibility of cAMP response after GIP washout from the cells. For this purpose, cAMP was measured by FRET using Epac1-Camps cytosolic sensor [22] on cells which were stimulated with 10 nM GIP and then superperfused with buffer to wash out GIP from the cells. Stimulation by GIP for 2 or 5 min caused rapid decreases of FRET ratio accounting for cAMP production and GIP washout did not result in significant FRET changes indicating irreversibility of cAMP response (Fig. 4A). In contrast, similar FRET measurements carried out on cells treated with Dyno-4a to block GIPR internalization, revealed that after the decrease of FRET signal in response to GIP stimulation, GIP washout resulted in an important return of FRET signal accounting for reversibility of cAMP response (Fig. 4B). Quantitative analysis of the data indicates that cAMP response was reversible in cells having GIPR internalization blocked whereas cAMP response was only weakly reversible in control cells internalizing GIPR (Fig. 4C).

To further assess sustained GIP-induced cAMP production, we measured activity of protein kinase A (PKA), a key effector for cAMP signaling, using AKAR3, a FRET sensor of PKA activity. As illustrated on Fig. 5A, stimulation by GIP for 2 min caused rapid increase of FRET, and GIP washout did not result in FRET decrease. This indicates that PKA was rapidly activated by GIP and kinase activity remained stable after GIP washout. In contrast, on cells

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treated with Dyngo-4a, FRET signal in response to GIP stimulation returned to basal value after GIP washout (Fig. 5A). Hence, PKA activity resulting from transient stimulation of GIPR, presumably at the plasma membrane, was reversible. Quantitative analysis of the data confirms that PKA activity was reversible in cells having GIPR internalization blocked whereas it was only weakly reversible in cells internalizing GIPR (Fig. 5B).

Control experiments were carried out in order to check inhibitory action of Dyngo-4a on GIPR internalization. Confocal microscopy imaging experiments showed a dramatic decrease of Flp-In™ HEK-GIPR cell membrane labeling by DY647-GIP in the presence of Dyngo-4a (not shown). This observation, which could be due to quenching of DY647-fluorescence in the presence of Dyngo-4a precluded the use of DY647-GIP to assess inhibitory
Inhibition of GIP receptor internalization affected the sustained phase of GIP-stimulated cAMP production. HEK cells expressing the human GIP receptor (Flp-In™/GIPR-293 cells) transfected with plasmids encoding BRET biosensor, Rluc-Epac1-citrine, were stimulated with GIP (10 or 100 nM) for BRET measurement of cAMP, as described in "Materials and Methods" section. Blue records (control) correspond to 1/BRET signal from experiments performed in the absence of inhibitor of internalization, and red records correspond to 1/BRET signals in the presence of inhibitor of internalization (Dyngo-4a, 60 μM). 1/BRET values expressed as percent of maximum in control assay are the mean ± SEM of 3 individual experiments. They account for a decrease of the sustained and late phase of cAMP production in the presence of Dyngo-4a. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Collectively, these results and controls strongly support the view that internalized GIPR contributes to the sustained phase and irreversibility of cAMP and PKA responses in Flp-In™ HEK-GIPR cells.

3.2. Accumulation of early endosomes containing GIPR enhanced the sustained phase of cAMP production

In order to further establish the relationship between the presence of GIPR in early endosomes and cAMP production profiles, we over-expressed a dominant negative of Rab7 in Flp-In™ HEK-GIPR. Rab7 is a GTPase that, in addition to its regulatory role in cargo transport from late endosomes to lysosomes, is required for early sorting events (Fig. 1). Dominant negative of Rab7 (DN-Rab7), by inhibiting exchange between Rab5 and Rab7 in early endosomes leads to accumulation of early endosomes in cells [27]. Accordingly, stimulation of cells over-expressing DsRed-DN-Rab7 by DY647-GIP resulted in strong accumulation of DY647-GIP-bound GIPR in endocytic vesicles positive for GFP-EEA1 (Fig. 7A, B). As a quantitative proof of early endosome accumulation, image analysis and quantification indicated that 88 ± 3% of endocytic vesicles containing DY647-GIP-bound GIPR were positive for EEA1 in cells over-expressing DsRed-DN-Rab7 after 30 min of stimulation. This co-localization level was 73 ± 16% in cells not expressing DsRed-DN-Rab7. After 60 min of stimulation, 81 ± 3% of endocytic vesicles containing DY647-GIP-bound GIPR were positive for EEA1 in cells over-expressing DsRed-DN-Rab7 whereas this co-localization level dropped to 24 ± 4% in cells not expressing DsRed-DN-Rab7 (Fig. 7C). These data were confirmed by determination of co-localization using Mander’s coefficient (not shown). We then characterized the kinetic of cAMP production in Flp-In™ HEK-GIPR cells transfected with plasmid encoding DsRed-DN-Rab7 and which, according to fluorescence confocal microscopy observations, over-expressed the encoded protein. As shown on Fig. 7D, after a rapid rise, level of cAMP was stabilized (for 1 or 10 nM GIP stimulation) or slightly declined during few minutes (for 100 nM GIP stimulation) and then increased continuously. These profiles of enhanced cAMP production in cells over-expressing DsRed-DN-Rab7 were observed for both physiological and pharmacological concentrations of GIP, namely from 1 nM to 100 nM GIP. Control experiments showed no significant modification of forskolin-stimulated cAMP levels in cells over-expressing DsRed-DN-Rab7 (Fig. 7E). Therefore, an accumulation of DY647-GIP-bound GIPR in early endosomes correlated with a continuous increase of cAMP level in Flp-In™ HEK-GIPR cells.

3.3. Identification of the active form of Gαs in early endosomes containing activated GIPR

At this step of the work, the view that internalized GIPR continues to stimulate cAMP production in early endosomes is based on data showing modulation of kinetic and level of cAMP by Dyngo-4a and Rab7 dominant negative acting respectively on internalization and accumulation of GIPR in early endosomes. Therefore, our next aim was to provide more direct evidence by detecting GIPR activation and signaling in early endosomes. We first used a confornational biosensor (termed GFP-Nb37) composed of a single-domain antibody (nanobody) tagged with GFP and recognizing Gαs only in its active form. In the absence of GIPR stimulation, genetically expressed GFP-Nb37 localized exclusively in the cytoplasm of cells (Fig. 8A). Immediately after stimulation by GIP, GFP-Nb37 was recruited to the plasma membrane. This recruitment of GFP-Nb37 to the plasma membrane lasted during the chronic stimulation by GIP (Fig. 8A). Strikingly, many endocytic vesicles containing DY647-GIP were also labeled by GFP-Nb37, demonstrating presence of active Gαs together with DY647-GIP-bound GIPR in these vesicles (Fig. 8A, lower images). Furthermore, vesicles positive for GFP-Nb37 also expressed DsRed-Rab5 or GFP-EEA1, two markers of early endosomes. This co-localization was illustrated by line scan analysis of confocal microscopy images of an endocytic vesicle (Fig. 8C). Quantification of co-localization indicated that 76.0 ± 7.0% of endocytic vesicles containing DY647-GIP-bound GIPR were labeled with GFP-Nb37 at time 30 min. This proportion decreased to 48.9 ± 7.9% at 60 min. In cells over-expressing DsRed-DN-Rab7, the proportion of endocytic vesicles labeled with Nb-37-GFP remained high, 82.8 ± 4.1% and 78.5 ± 4.7% at times 30 and 60 min, respectively (Fig. 8D, E). From these different experiments, it appears that active Gαs is present together with activated GIPR in early endocytic vesicles and over-expression of Rab7 dominant negative prolongs this co-localization.
3.4. Direct identification of cAMP production on early endosomes containing internalized GIPR

To further demonstrate production of cAMP by internalized GIPR, we performed detection of cAMP in endosomes. For this purpose, we took advantage of the fact that PtdIns(3)P is the most abundant phosphoinositide in the membrane of early endosomes to target expression of a FRET cAMP probe at the external surface of early endosomes using FYVE domain of EEA1. The FYVE domain is a protein motif that allows the interaction of cytosolic proteins, A, control

![Graph A](image1)

B, Dyngo-4a

![Graph B](image2)

C, reversibility

![Graph C](image3)

**Fig. 4.** Inhibition of GIPR internalization abolished irreversibility of GIP-induced cAMP production. Stable Flp-In®GIPR-293 cells were transfected with plasmid encoding cytosolic biosensor Epac1-camps. 24 h post-transfection, cells were washed and then incubated with medium (A, control) or with medium containing 30 μM Dyngo-4a (B, Dyngo-4a). Next, cells were superfused with 10nM GIP for 2 or 5 min with subsequent washout. All experiments were performed at 25 °C. During these experiments, FRET was recorded as described in “Materials and Methods” section. Four representative records are shown. Values of YFP/CFP FRET ratios are expressed relative to initial FRET ratio which was normalized to 1.00. For analysis of reversibility of cAMP response after GIP washout, (C, reversibility), signal reversibility calculated by setting the minimum FRET ratio value equal and the initial FRET value before GIP stimulation to 100%. Values are the mean ± SEM of 8–10 records (noticed on figure). The data indicate that cAMP response was reversible in cells having GIPR internalization blocked whereas cAMP response was only weakly reversible in control cells internalizing GIPR.
Fig. 5. Inhibition of GIPR internalization abolished reversibility of GIP-induced PKA activation. Stable Flp-In™GIPR-293 cells were transfected with plasmid encoding PKA biosensor AKAR3. 24 h post-transfection, cells were washed and then incubated with medium (control curve) or with medium containing 30 μM Dyngo-4a (Dyngo-4a curve). Next, cells were superfused with 10 nM GIP for 2 min with subsequent washout. All experiments were performed at 25 °C. During these experiments, FRET was recorded as described in “Materials and Methods” section. Two representative records are shown. Values of YFP/CFP FRET ratios are expressed relative to initial FRET ratio which was normalized to 1.00. For analysis of reversibility of PKA response after GIP washout, (panel B), signal reversibility calculated by setting the minimum FRET ratio value equal and the initial FRET value before GIP stimulation to 100%. Values are the mean ± SEM of 8 records (noticed on figure). The data indicate that PKA activity was reversible in cells having GIPR internalization blocked whereas PKA activity was only weakly reversible in control cells internalizing GIPR.

Fig. 6. Dyngo4-a efficiently inhibited GIPR internalization and did not affect GIP binding and forskolin-stimulated cAMP production. HEK T cells transiently expressing the GFP-tagged GIPR (termed GFP-GIPR) were stimulated with 1 μM GIP at 37 °C with or without 30 μM Dyngo-4a, an inhibitor of dynamin-dependent internalization. Confocal microscopy images were recorded at various times of stimulation. Panel A corresponds to images of representative microscopy field at times 15 and 60 min of stimulation, illustrating kinetic of GIP-stimulated internalization of GFP-GIPR (upper images) which is inhibited by Dyngo-4a (lower images). In panels B and C, Flp-In™GIPR-293 cells were incubated with 125I-Phe1-GIP with or without 1 μM GIP (for non specific binding determination) in the presence or absence of 30 μM Dyngo-4a. Specific 125I-Phe1-GIP binding was determined at various times of incubation (panel B) or Specific 125I-Phe1-GIP binding at 30 min of incubation was inhibited by increasing concentrations of GIP (panel C). Results are expressed as percent of maximum specific binding and values are mean ± SEM of 3 individual determinations. They indicate that Dyngo-4a did not significantly influence kinetic and affinity of GIP binding. Panels D and E show representative experiments of cAMP measurement by BRET carried out on Flp-In™GIPR-293 cells transfected with plasmid encoding Rluc-Epac1-Citrine and stimulated by Forskolin (10 μM) in the presence or the absence of Dyngo-4a (30 or 60 μM). Values of 1/BRET were expressed as percent of maximum.
Fig. 7. Accumulation of activated internalized GIP receptors in early endosomes enhanced the sustained phase of cAMP production. Confocal microscopy images captured after 60 min of stimulation with 100 nM DY647-GIP of HEK cells expressing the human GIP receptor (Flp-In®GIPR-293 cells) co-transfected with plasmid encoding GFP-EEA1 (displayed in green) plus plasmid encoding DsRed tagged dominant negative of Rab7 (DsRed-DN-Rab7, displayed in blue, panel A), or with plasmid encoding GFP-EEA1 plus empty plasmid pcDNA5 (control, panel B). Pearson's correlation coefficients –PCC- are indicated on images. Presence of DY647-GIP-labeled GIPR in early endosomes was quantified by analyzing co-localization of DY647-GIP and GFP-EEA1. Results at times 30 and 60 min, reported in panel C, show persistent co-localization at time 60 min in cells overexpressing DsRed-DN-Rab7, whereas a dramatic decrease is seen in control cells not transfected with DsRed-DN-Rab7 (p < 0.001). These data were confirmed by determination of co-localization using Mander's coefficient (not shown). In panel D, Flp-In®GIPR-293 cells were co-transfected with plasmid encoding BRET biosensor (Rluc-Epac1-citrine) plus plasmid encoding DsRed-DN-Rab7 or empty plasmid PcDNA5 as control. Cells were stimulated with GIP (10 or 100 nM) for BRET measurement of cAMP, as described in “Materials and Methods” section. Blue records correspond to 1/BRET signal ratio from control cells and Red records correspond to 1/BRET signal ratio from cells over-expressing DsRed-DN-Rab7. 1/BRET values expressed as percent of maximum in control assay are the mean ± SEM of 3 individual experiments. They account for an increase of the sustained and late phase of cAMP production in the presence of DN-Rab7. In panel E, results indicate that over-expression of dominant negative of Rab7 does not modify forskolin-induced production of cAMP. Flp-In®GIPR-293 cells co-transfected with plasmid encoding Rluc-Epac1-Citrine and DsRed-DN-Rab7 or pcDNA5 (control) were stimulated with 10 μM forskolin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 8. Immunological detection of active Gs in endosomes containing activated GIP receptor. HEK cells expressing the human GIP receptor (Flp-In<sup>®</sup>GIPR-293 cells) transfected with plasmid encoding the GFP-tagged nanobody specifically recognizing the active form of Gs (termed GFP-Nb37, displayed in green) were stimulated with DY647-GIP. Fluorescence confocal microscopy images were captured at various times of stimulation. Images in panel A show co-localization of DY647-GIP, DsRed-Rab5 and GFP-Nb37 in endocytic vesicles. The white arrow on GFP-Nb37 images shows membrane recruitment of GFP-Nb37. A merge image of DY647-GIP and GFP-Nb37 co-locates is shown for the time 30 min of stimulation with DY647-GIP. It can be appreciated from an image zoom of the cell that many endosomes labeled by DY647-GIP are also labeled by the antibody against active Gs, GFP-Nb37 (displayed by white arrows). In panels B and C are shown an image zoom of an endosome and the line scan, respectively. Panel D, confocal microscopy images of Flp-In<sup>®</sup>GIPR-293 cells co-transfected with plasmid encoding GFP-NB37, (displayed in green) and DsRed-DN-Rab7 (displayed in blue) at time 60 min of stimulation with 100 nM DY647-GIP. Upper images correspond to cells co-transfected with plasmids encoding GFP-NB37 and DsRed-DN-Rab7 whereas lower images correspond to control cells co-transfected with plasmids encoding GFP-NB37 and pcDNA5. Panel E reports quantification of co-localization indicating that 76.0 ± 7.0% of endocytic vesicles containing DY647-GIP-bound GIPR were labeled with GFP-Nb37 at time 30 min. This ratio decreased to 48.9 ± 7.9% at 60 min. In cells over-expressing DsRed-DN-Rab7, the ratio of endocytic vesicles labeled with Nb-37-GFP was high, 82.8 ± 4.1% and 78.5 ± 4.7% at times 30 and 60 min, respectively. Co-localization ratios at 60 min were significantly different in control cells and in cells over-expressing DsRed-DN-Rab7, p < 0.002. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
such as EEA1, with membranes containing the lipid phosphatidylinositol 3-phosphate [28]. The probe for cAMP detection was thus a FRET probe consisting in EYFP-Epac1 (binding domain)-ECFP-EAE1 (FYVE domain). This probe (termed FYVE-EPAC) was efficiently targeted to the membrane of endosomes in the majority of cells (Fig. 9). In order to determine if internalized GIPR continued to stimulate production of cAMP, we compared FRET ratio in early endosomes containing DY647-GIP-bound GIPR which therefore contained activated GIPR, with FRET ratio in pre-existing endosomes lacking DY647-GIP from the same cell. As an example, on Fig. 9B, are outlined images of 4 endosomes from a single cell stimulated with DY647-GIP showing that FRET ratios were 2.60 and 2.67 at the surface of the two endosomes containing DY647-GIP-bound GIPR whereas FRET ratios were 2.91 and 3.00 at the surface of the two endosomes lacking DY647-GIP-bound GIPR. The lower FRET ratio at the surface of endosomes containing DY647-GIP-bound GIPR most likely reflected endosomal production of cAMP. Indeed, the 4 endosomes were equally distant to the cell surface and were equally subject to diffusible cAMP produced at the plasma membrane. Furthermore, analysis of a large population of endosomes from 13 individual cells stimulated with DY647-GIP indicated that in each cell (represented by an empty circle linked by a dotted line to an empty square), FRET ratio at the membrane surface of early endosomes containing activated GIPR was lower than FRET ratio in early endosomes lacking activated GIPR (Fig. 9C). Averaged FRET ratios were 2.56 ± 0.03 versus 2.83 ± 0.04, p < 0.001 at time 30 min of stimulation. These data unequivocally establish the existence of production of cAMP at the surface of endosomes containing both GIPR and its ligand.

4. Discussion

The current work provides a series of experimental data demonstrating that, once internalized and located in the membrane of early endosomes, GIPR continues to be activated and to trigger production of cAMP. Direct evidence for the activity of internalized GIPR is based on identification of the active form of Gαs in early endosomes containing GIP using a genetically encoded GFP tagged nanobody, and on detection of a distinct FRET signal accounting for cAMP production at the surface of endosomes containing GIP compared to endosomes without GIP. Furthermore, strong indirect experimental proofs were obtained on cells treated with a pharmacological inhibitor of GIPR internalization, such as...
decrease of the sustained phase of cAMP and PKA kinetics and reversibility of responses following GIP washout. Furthermore a continuous increase of cAMP levels over time was observed in cells expressing genetically encoded dominant-negative of Rab7 which causes accumulation of early endosomes.

In fact, direct identification of active G\(\alpha\)s and CAMP production at the surface of endosomes was made possible by the combinational use of three major biological tools: the fluorescent agonist of the GIPR, DY647-GIP, the fluorescent nanobody recognizing active G\(\alpha\)s and FYVE-EPAC-based FRET sensor for local endosomal cAMP production. DY647-GIP, which retains biological activity of native GIP and which fully co-localizes with GIPR for at least the first 2 h of post-endocytic trafficking [18], enabled to distinguish endosomes containing activated GIPR from endosomes devoid of fluorescent GIP and therefore, not containing activated GIPR. The second crucial tool was the GFP-tagged nanobody, GFP-Nb37. It specifically recognizes the guanine-nucleotide-free form of G\(\alpha\)s representing the catalytic intermediate of G\(\alpha\)s activation [9]. The third biological tool, especially developed for this study, was FYVE-EPAC FRET sensor for CAMP which was specifically targeted to early endosomes by insertion of FYVE sequence. Indeed, FYVE sequence, derived from EEA1, provided binding of the FRET sensor to PtdIns(3)P contained in the membrane of early endosomes [28].

Our findings contradict the classical concept whereby G-protein dependent signaling of GPCRs occurs exclusively at the plasma membrane and that internalized GPCRs are silent with respect to this signaling pathway. On the other hand, based on these findings, the GIPR joins the group of GPCRs for which endosomal G-protein couple signaling have been suggested or demonstrated. So far, this group of GPCRs includes Parathyroid Hormone receptor, Thyroid-Stimulating Hormone receptor, Vasopressin 2 receptor, dopamine D1 receptor, Sphingosine-1-phosphate receptor, \(\beta_2\)-adrenergic receptor, Pituitary Adenylate Cyclase 1 receptor and Glucagon-like peptide 1 receptor [5–9,11,29,30]. In the current study, kinetic profiles of cAMP production and PKA activity support the view that CAMP signaling resulting from plasma membrane GIPR is subject to desensitization whereas endosomal CAMP signaling is more sustained. How internalized GIPR triggers G-protein dependent signaling in endosomes and how this spatio-temporal signaling can be reconciled with sequential events governing GIPR activation-desensitization (inactivation) cycle at the plasma membrane are important and timely issues which are not elucidated yet. In the canonical cycle of Gs-coupled GPCR activation and desensitization, the agonist ligand binds to an active form of the receptor and stabilizes it, permitting coupling to cognate heterotrimeric GDP-bound G proteins. The active receptor-G-protein complex catalyzes GDP-GTP exchange causing dissociation of the G\(\alpha\)x subunit and \(\beta\gamma\) dimer from the receptor. Next, GTP-bound G\(\alpha\)x binds to and activates adenylate cyclase that converts ATP into cAMP. After G-protein coupling/decoupling, the receptor is phosphorylated by GRK and by second messenger-dependent kinases (such as PKA), an event, which most often causes recruitment of the adaptor proteins \(\beta\)-arrestins. \(\beta\)-arrestins terminate G-protein-dependent signal and permit the targeting of the receptor to clathrin-coated pits for endocytosis. At this step, the receptor is associated with a set of proteins of internalization and trafficking cell machineries. Studies with \(\beta_2\)-adrenergic receptor support the view that, after dissociation of G\(\alpha\)x from the membrane receptor and recruitment of \(\beta\)-arrestins, G\(\alpha\)x is activated in endosomes containing \(\beta_2\)-adrenergic receptor presumably free of \(\beta\)-arrestins [9]. A different mechanism has been elucidated for the PTH and vasopressin 2 receptors in which agonist-activated receptors form a ternary complex that includes arrestins and G\(\alpha\)x\(\gamma\) dimer which accelerate rate of G\(\alpha\)x activation and increase levels of activated G\(\alpha\)x, leading to persistent production of cAMP. In this latter example, arrestins play the role of scaffolding protein for maintaining activated G\(\alpha\)s associated with the receptors [8,31]. Very recent, identification of maga-complexes containing a GPCR simultaneously engaged with a G-protein and \(\beta\)-arrestin bound to phosphorylated C-terminal tail of the receptor, provided physical and biochemical rationale for the existence of functional GPCR complexes in endosomes [32]. In the current case of GIPR, it is unlikely that \(\beta\)-arrestins could play a role in activation of G\(\alpha\)s. Indeed, we showed that the GIPR does not recruit \(\beta\)-arrestins upon activation [18,19] and GIP tagged \(\beta\)-arrestin-1 or -2 were never identified in endosomes containing GIPR [18]. Hence, the molecular partners of endosomal G-dependent signaling of the GIPR, as well as precise mechanism of plasma membrane GIPR desensitization, remain to be identified.

Another difference between GIPR and PTHR concerns the influence of endosome acidification for the termination of endosomal cAMP production. Indeed, we did not observe any significant effect of bafilomycin, an inhibitor of v-ATPase, on the sustained phase of cAMP production (not illustrated), whereas, for the PTH receptor, based on results with this inhibitor, it has been proposed that endosomal cAMP production is turned off by endosome acidification which dissociates PTH from its receptor. On the other hand, as for GIPR in the current study, TRHR was shown to bind TSH and be activated in acidic conditions [6].

In line with presence of active G\(\alpha\)s in endosomes containing activated GPCRs, a recent study shows that presence of G\(\alpha\)s in endosomes has a function which is not restricted to stimulation of diffusible second messengers. Indeed, endosomal G\(\alpha\)s was found to contribute to the endocytic sorting of several GPCRs to lysosomes. This occurs through binding to GPCR-associated binding protein–1 (GASP1) and dysbindin, two key proteins of the endosomal sorting of GPCRs in intra-luminal vesicles of multivesicular bodies [33]. Interestingly, this function of G\(\alpha\)s in lysosomal degradation of GPCRs does not require its activation, supporting a scaffolding rather a signaling function. In light of these findings, it is plausible that during post-endocytic sorting of GPCRs, G\(\alpha\)s successively plays a role in their endosomal signaling in early endosomes and then, in their trafficking to lysosomes.

Results from the current study on HEK cells represent a real milestone and call future works on cells naturally expressing GIPR. Indeed, a major issue raised by endosomal cAMP signaling lies in its physiological relevance. In fact, the novelty of the discovery that GPCRs continue to stimulate cAMP formation on endosomes is enhanced by accumulating data demonstrating that cAMP is compartmentalized in cells to enable discrete pathways of localized signaling and related physiological responses to occur [34]. In this regard, it can be hypothesized that endosomal cAMP production mediated by internalized GPCRs contributes to the spatio-temporal organization of cAMP signaling cascade involving PKAs, EPACs, AKAPs, PDEs, etc.

As an example supporting this concept, it was reported that internalization of the TSH receptor is required to ensure normal actin rearrangement in thyroid follicles and phosphorylation of vasodilator-stimulated phosphoprotein (VASP) [6]. As another example, the duration of PTH-induced calcemic and phosphate responses in mice which are dependent of PTH ligands, was related to the duration of cAMP responses in correspondence with the ability of the ligands to stimulate cAMP from internalized PTH receptors [5]. The divergent antinatriuretic and antidiuretic actions of vasopressin and oxytocin, both acting on V2 vasopressin receptor, were also explained by different profiles of cAMP responses to the two hormones, with vasopressin causing an endosomal sustained cAMP production and oxytocin causing a short cAMP production, limited to the plasma membrane [8]. Moreover, a relationship between expression of cAMP-dependent genes and endosomal cAMP production triggered by internalized \(\beta\)-adrenergic receptor was recently reported [12]. In this study, inhibitors of \(\beta\)-
adrenergic receptor internalization reduced PKA phosphorylation of CREB and expression of several cAMP-regulated genes, including the gene encoding the phosphoenolpyruvate carboxykinase 1. In light to these data, our forthcoming investigations should address the question of how endosomal GIP-induced cAMP signaling is required for full physiological functions of GIP. Such a challenging question requires development of new relevant biological tools such as endocrine pancreatic β-cells expressing the GIPR at a physiological level.

In conclusion, we have demonstrated that, once internalized and located in the membrane of early endosomes, the receptor for Glucose-dependent Insulinotropic receptor (GIPR) continues to trigger production of cAMP which accounts for sustained cAMP production and PKA activation. Hence, production of endosomal cAMP by internalized presumably contributes to the spatio-temporal organization of cAMP signaling cascade downstream GIPR.

Conflict of interest

The authors declare have no conflict of interest to declare.

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