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

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ABSTRACT

Until very recently, G-protein dependent signal of GPCRs 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.

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 exclusively originate from the cell plasma membrane, where embedded GPCRs bound their cognate ligands and are subsequently activated to stimulate production of second messengers (3′,5′-cyclic adenosine monophosphate, cAMP or inositol triphosphate, IP3) and downstream signaling cascade [3]. On the other hand, activation of membrane GPCRs is generally immediately followed by their Ser/Thr phosphorylation by second messenger-dependent kinases and G-protein Receptor Kinases (termed GRKs) [1–4]. Phosphorylated GPCRs then recruit adaptor proteins such as β-arrestins which uncouple G-proteins from membrane receptors and promote GPCR endocytosis. Alternatively, activated and phosphorylated GPCRs can interact directly with components of the endocytic machinery which generally involves clathrin and the clathrin adaptor AP2. Coated-pits containing GPCRs are then separated from the plasma membrane by action of dynamin, leading to the formation of early endosomes. Finally, internalized GPCRs are sorted *via* the endocytic pathway to lysosomes or are recycled to the cell surface [1–4]. Thus, consensus holds that internalization disrupts G-protein-dependent signaling of GPCRs and consequently, internalized GPCRs become silent with respect to G-protein dependent production of diffusible second messengers.

Recently, the classical concept whereby G-protein-dependent signaling of GPCRs is restricted to plasma membrane has been

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 β_2-adrenergic receptor in which conformational biosensors, consisting of nanobodies, enabled identification, by confocal microscopy, of the active form of the β_2-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 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 of Gs 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 present of 100-fold excess of unlabeled peptide). Radio-labeled GIP was obtained by radio-iodination of Phe^1-GIP(1–30) with ^{125}I-Na (Perkin Elmer, France) in the presence of chloramine T and was HPLC purified on a C-18 column. ^{125}I-Phe^1-GIP bound to a single class of GIPR binding sites from HEK 293T or Flp-In™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: Dyngo-4a from Abcam (Cambridge, UK), Forskolin from Sigma-Aldrich (St. Quentin Fallavier, France).

Plasmids encoding DsRed tagged Rab5 (termed DsRed-Rab5), DsRed tagged Rab7-DN (termed DsRed-Rab7-DN), GFP tagged EEA1 (termed GFP-EEA1) were supplied by Addgene (www.addgene.org). Plasmid for BRET measurements of cAMP production, namely *R*luc-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 TGC CCT TGG TGG ATT TCT TCT GCT GGC AAT CTA GTC AAC GG and aaa ctc gag ttatccttgcaagtcattgaaaca, and inserted *via* BamHI and XhoI restriction sites into the Epac1-camps vector backbone. The resultant targeted sensor contained the full Epac1-camps sequences followed by a flexible linker GSMPLVDFFC and the FYVE sequence from WQSSQ on. Plasmid to detect PKA activity encoded A Kinase Activity Reporter (AKAR3) sensor. It was constructed on the basis of previous report [23]. Plasmid encoding the nanobody Nb-37 recognizing activated form of Gs was generated according to [24]. Nb-37 specifically recognizes nucleotide-free form of α subunit of Gs [9].

2.2. Cell lines and transfections

HEK 293 cells stably expressing the GIPR (Flp-In™ HEK-GIPR) were obtained using the Flp-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% CO_2. Transfections for BRET experiments were performed using polyethylenimine (PEI) transfection reagent (1 mg/mL, pH 7.4) (Polyplus, 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 Lipofectamin 2000 (Invitrogen Life Technologies) following provider's instructions (ratio DNA (μg)/LPF2000 (μL) 1:2).

2.3. BRET assay of cAMP production

Flp-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 30 s for 60 min. Luminescence and fluorescence readings were performed on a Mithras LB940 instru-