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MOLECULAR METABOLISM

Gαs regulates Glucagon-Like Peptide 1 Receptor-mediated cyclic AMP generation at Rab5 endosomal compartment

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ABSTRACT

Objective: Upon activation, G protein coupled receptors (GPCRs) associate with heterotrimeric G proteins at the plasma membrane to initiate second messenger signaling. Subsequently, the activated receptor experiences desensitization, internalization, and recycling back to the plasma membrane, or it undergoes lysosomal degradation. Recent reports highlight specific cases of persistent cyclic AMP generation by internalized GPCRs, although the functional significance and mechanistic details remain to be defined. Cyclic AMP generation from internalized Glucagon-Like Peptide-1 Receptor (GLP-1R) has previously been reported from our laboratory. This study aimed at deciphering the molecular mechanism by which internalized GLP-R supports sustained cyclic AMP generation upon receptor activation in pancreatic beta cells.

Methods: We studied the time course of cyclic AMP generation following GLP-1R activation with particular emphasis on defining the location where cyclic AMP is generated. Detection involved a novel GLP-1 conjugate coupled with immunofluorescence using specific endosomal markers. Finally, we employed co-immunoprecipitation as well as immunofluorescence to assess the protein—protein interactions that regulate GLP-1R mediated cyclic AMP generation at endosomes.

Results: Our data reveal that prolonged association of G protein α subunit Gαs with activated GLP-1R contributed to sustained cyclic AMP generation at Rab5 endosomal compartment.

Conclusions: The findings provide the mechanism of endosomal cyclic AMP generation following GLP-1R activation. We identified the specific compartment that serves as an organizing center to generate endosomal cyclic AMP by internalized activated receptor complex.

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Keywords GLP-1 receptor; Rab5; Cyclic AMP; Insulin secretion; Gαs; Beta arrestin-1; Pancreatic beta cells

1. INTRODUCTION

G protein coupled-receptor (GPCR) signaling is initiated through agonist mediated stabilization of the receptor in active conformation resulting in association with heterotrimeric G proteins, which upon guanine nucleotide exchange initiates diverse cellular processes [1,2]. In the canonical pathway of GPCR activation, association of GPCR with heterotrimeric G proteins takes place exclusively at the plasma membrane [2,3]. This interaction is terminated by a series of events involving phosphorylation of GPCRs by G protein receptor kinases (GRKs) and recruitment of β arrestins which sterically hinder the association of GPCR with G protein α subunit to desensitize and internalize the receptor [4—6]. The establishment of the recent model of sustained signaling by a number of GPCRs [7—14], which continue signaling after initial agonist activation necessitates reassessment of the role of these regulatory proteins in mediating the GPCR response.

Endosomal localization of Galpha S subunit (Gαs) and its role in endosomal receptor trafficking and signaling is well documented [11,15—18]. Gαs has been reported to be activated and signal from the intracellular compartment following cell-surface activation and internalization of parathyroid hormone receptor (PTHR) [19], β2-adrenergic receptor (β2AR) [11] and vasopressin type 2 receptor (V2R) [16]. Single—particle electron microscopy analysis revealed simultaneous association of G protein α subunit and beta arrestin at the C terminal tail of Class B GPCRs forming megaplexes to provide the physical basis of interaction among these GPCR regulatory proteins [20] in regulating receptor internalization as well as endogenous cyclic AMP formation. In addition, Rosciglione et al. reported interaction of Gαs with ESCRT (endosomal sorting complex required for transport), highlighting its role in cellular trafficking of GPCRs [21].

GLP-1R mediated generation of cyclic AMP had previously been reported from our laboratory [22]. The internalized activated receptor complex was associated with Bodipy Forskolin, and pharmacological inhibition of internalization was found to attenuate GLP-1R mediated cyclic AMP generation in BRIN-BD11 pancreatic beta cells [22]. In the present study, we report the mechanism by which GLP-1R activation resulted in the generation of cyclic AMP from endosomes. Our data reveal that GLP-1R remained associated with Gαs after internalization

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of the receptor, which, in turn, contributes to sustained cyclic AMP generation upon GLP-1R activation.

2. MATERIALS & METHODS

2.1. Reagents

BRIN-BD11 was procured from European Collection of Cell Cultures (ECACC cat no: 10033003). RPMI Glutamax, Lipofectamine 2000, penicillin-streptomycin, gentamycin and sodium pyruvate were purchased from Thermo-Scientific, the mRFP-Rab5A was a gift from Ari Helenius (Addgene plasmid # 14437) [23], Myc-Rab5A:S34N was a gift from Qing Zhong (Addgene plasmid # 28044) [24], Gαs-GFP was a kind gift from Marc Rasenick [25] and beta arrestin-1 plasmid, was a gift from Robert Lefkowitz (Addgene plasmid # 14687&42196) [26]. Rabbit monoclonal antibody against Rab5 was purchased from Cell Signaling Technology, MA, USA; mouse monoclonal antibody against Gαs (Gαs/olf (sc-365855)) was purchased from Santacruz Biotech, Texas, USA; anti rabbit secondary antibody coupled to Alexa 594 (Life Technologies) was purchased from Thermo-Fischer Scientific and anti-mouse secondary antibody coupled to FITC was purchased from Sigma. Exendin-4 was obtained from Prof. DiMarchi's laboratory. All other chemicals used in this study were of reagent grade.

2.2. Cell culture

BRIN-BD11 pancreatic beta cells were cultured at 37° Celsius with 5% CO_2 in RPMI media supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1 mM Sodium pyruvate, 10 μg/ml Gentamycin, 100 units/ml Penicillin, and 100 μg/ml Streptomycin following the protocol published earlier from our laboratory [22].

2.3. Time course of GLP-1R — mediated cyclic AMP generation

A time-course of cyclic AMP generation was carried out in BRIN-BD11 pancreatic beta cells using Exendin-4 (100 nM), Liraglutide (10 nM) and GLP-S-S tetramethyl rhodamine conjugate (1 μM) using direct cyclic AMP ELISA kit (Enzo Life Sciences-ADI-900-163). The cells were seeded in 24 well plates and prior to ligand treatment, they were incubated with Kreb Ringer's Buffer (KRB) containing 0.2% BSA for 1 h. Cells were treated for with ligands for different time points at indicated concentrations in KRB media containing IBMX (200 μM). After treatment, the media was removed and fresh KRB media containing IBMX (200 μM) was added. At respective time points, the cells were lysed with the addition of 0.1N HCl and processed for cyclic AMP estimation through ELISA determination, following kit protocol.

2.4. CRE luciferase reporter assay

GLP-1R mediated signaling was assessed by cyclic AMP responsive element (CRE) Luciferase reporter assay following the method of Fortin et al. [27] with modifications. The cells were grown in 70 mm dish until they attain 70% confluence. A cAMP responsive element-luciferase reporter plasmid encoding the luciferase reporter gene under the control of minimal promoter and six tandem repeats of CRE transcriptional response element (CRE6X-luc) [22] and a beta galactosidase plasmid were transiently transfected in 1:1 ratio using Lipofectamine 2000, following manufacturer's protocol. Four hours post transfection, cells were transferred to 96-well Cell Bind plates (Corning) at a density of 50,000 cells per well. After 24 h, cells were treated with and without appropriate GLP-1R agonist in complete medium for another 4 h. The medium was then aspirated, cells were lysed, and luciferase activity was measured using Steadylite plus reagent (Perkin Elmer Life and Analytical Science, Waltham, MA). Correction for inter-well variability of transfection was carried out through β-galactosidase assay by addition of 2-nitrophenyl-beta galacto pyranoside (Sigma). After incubation for 15 min at 37 °C, substrate cleavage was quantified by measuring optical density at 405 nm in ELISA plate reader (Perkin Elmer, USA), and the corresponding values were used to normalize luciferase activity. The data were expressed as fold luciferase activity increase on agonist treatment over untreated control, which is considered as basal.

2.5. Immunocytochemical staining and confocal microscopy

The GLP-1 receptor-ligand internalization was conducted following the method of Kuna et al. [22]. Briefly, BRIN-BD11 cells were co-transfected with GLP-1R-GFP and beta arrestin-1 RFP plasmid using Lipofectamine 2000 (cat# 11668019, Thermo Fisher) and plated in six-well plates containing 22-mm-diameter glass coverslips. Sixty hours after transfection, cells in coverslips were incubated with Exendin-4 (100 nM) in 200 μL of Krebs-HEPES buffer for 60 min at 4 °C in the dark. Cells were then washed in phosphate buffer saline (PBS) and incubated at 37 °C for the desired time period in complete medium, after which they were fixed in 4% paraformaldehyde, mounted in Vectashield mounting medium (Vector Laboratories), and imaged using a Zeiss LSM 710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) equipped with krypton-argon laser sources. Pinhole diameter was maintained at 1 airy unit. Image acquisition was carried out using a 63× oil immersion objective lens with 2× optical zoom using the Zenlite 2011 program. To study the association between activated receptor complex and Gαs, the GLP-1Tmr (1 μM) was internalized following similar protocol and at different time points, cells fixed in 4% paraformaldehyde, permeabilized with 0.1% (w/v) Triton X-100 in PBS, incubated with Gαs antibody overnight and counterstained with goat anti mouse secondary antibody coupled to FITC. To evaluate the distribution of GLP-1R GFP and Gαs, BRIN-BD11 cells were transfected with GLP-1R-GFP using Lipofectamine 2000 and plated in six-well plates containing 22-mm-diameter glass coverslips. Sixty hours after transfection, cells in coverslips were incubated with Exendin-4 (100 nM) in 200 μL of Krebs-HEPES buffer for 60 min at 4 °C in the dark. The cells were then washed in PBS and incubated at 37 °C for the desired time period in complete medium after which they were fixed in 4% paraformaldehyde, permeabilized with 0.1% (w/v) Triton X-100 in PBS, incubated with Gαs antibody overnight, and counterstained with goat anti mouse secondary antibody coupled to Alexa 594. Association of Rab5 with Gαs and to GLP-1R-GFP was carried out following similar methods.

2.6. Image quantification

Image quantification was carried out in ImageJ (JACoP plugin) and co-localization was measured by Pearson Correlation Coefficient (PC) using the formula:

$$R_r = \left(\frac{\sum_i (Ch1_i - Ch1_{mean}) \cdot (Ch2_i - Ch2_{mean})}{\sqrt{\left\{ \left(\sum_i (Ch1_i - Ch1_{mean})^2 \right) \left(\sum_i (Ch2_i - Ch2_{mean})^2 \right) \right\}}} \right)$$

where Ch1 = channel 1 (green), Ch2 = channel 2 (red); values indicating co-localization (0.5—1.0); values indicating absence of co-localization (−1.0 to 0.5).

2.7. Cross-linking and co-immunoprecipitation

BRIN-BD11-GLP-1R GFP stable cell lines were grown to 85% confluency in 100 mm dishes. Cells washed with PBS were treated with or without Exendin-4 for 30 min in KRB media, and the cells were

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