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Real-time trafficking and signaling of the glucagon-like peptide-1 receptor ☆

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ABSTRACT

The glucagon-like peptide-1 incretin receptor (GLP-1R) of family B G protein-coupled receptors (GPCRs) is a major drug target in type-2-diabetes due to its regulatory effect on post-prandial blood-glucose levels. The mechanism(s) controlling GLP-1R mediated signaling are far from fully understood. A fundamental mechanism controlling the signaling capacity of GPCRs is the post-endocytic trafficking of receptors between recycling and degradative fates. Here, we combined microscopy with novel real-time assays to monitor both receptor trafficking and signaling in living cells. We find that the human GLP-1R internalizes rapidly and with similar kinetics in response to equipotent concentrations of GLP-1 and the stable GLP-1 analogues exendin-4 and liraglutide. Receptor internalization was confirmed in mouse pancreatic islets. GLP-1R is shown to be a recycling receptor with faster recycling rates mediated by GLP-1 as compared to exendin-4 and liraglutide. Furthermore, a prolonged cycling of ligand-activated GLP-1Rs was observed and is suggested to be correlated with a prolonged cAMP signal.

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1. Introduction

GLP-1[1] is a key incretin hormone that regulates post-prandial blood glucose levels. GLP-1 is secreted from the intestinal L-cells in response to food intake and activates the 7TM[2]/GPCR[3] GLP-1R[4] located on e.g. pancreatic β-cells. Activated GLP-1R then initiates several effects leading to an overall decrease in blood glucose levels, including i) increased insulin secretion in a glucose-dependent manner, an effect commonly known as the incretin effect, ii) decreased glucagon secretion, and iii) decreased food intake through satiety induction (Holst, 2007). These effects are diminished in type 2 diabetic patients, which has led to the development of GLP-1R agonists as anti type-2-diabetic drugs (Meier and Nauck, 2010). Since endogenous GLP-1 is quickly degraded by dipeptidyl peptidase-4 (DPP-4[5]) and cleared by the kidneys, long-acting GLP-1 analogues are used for treatment purposes. Two such analogues have been approved, exenatide and liraglutide (Meier, 2012).

The signaling cascades leading to insulin secretion via GLP-1R have been extensively studied (Mayo et al., 2003). However, the mechanism(s) involved in the termination of GLP-1R-mediated cellular processes remain largely unknown (Widmann et al., 1996a,b, 1997). One highly conserved mechanism that is important for terminating G protein-mediated signaling from many 7TM/GPCRs is the internalization/endocytosis and the subsequent intracellular sorting of the receptors. Specifically, following activation by a ligand, many 7TM/GPCRs are phosphorylated by GPCR kinases, bind β-arrestins and are internalized (Hanyaloglu and von Zastrow, 2008). Internalized receptors are then either recycled back to the

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[1] Glucagon-like peptide-1.
[2] Seven transmembrane-spanning.
[3] G protein-coupled receptor.
[4] Glucagon-like peptide-1 receptor.
[5] Dipeptidyl peptidase-4.

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cell surface where they can engage with ligands once again, or targeted for post-endocytic degradation, resulting in permanent signal termination from the receptor (Tsao et al., 2001; Whistler et al., 2002).

Both the rat and the human GLP-1Rs have been shown to rapidly internalize after activation by GLP-1 (Widmann et al., 1995; Jorgensen et al., 2007; Kuna et al., 2013). However, the kinetics of internalization as well as the post-endocytic sorting of GLP-1Rs in response to diverse ligands – including approved drugs – have not yet been studied in detail.

Here, we investigated the internalization and the signaling capacity of GLP-1R in real time in living cells using FRET[6] techniques in response to GLP-1, exendin-4 and liraglutide. In addition, we monitored the post-endocytic fate of GLP-1R and its ligands as well as confirmed the biological relevance of GLP-1R internalization in vivo using fluorescently labeled GLP-1 analogues.

2. Experimental procedures

2.1. Materials

Cell culture medium (DMEM) with glutaMAX™-I and 4.5 g/L D-glucose, Dulbecco's phosphate-buffered saline (DPBS) without $CaCl_2$ and $MgCl_2$, optimem®I with glutaMAX™-I, lipofectamine2000, hank's balanced salt solution (HBSS), alexa fluor® 594 goat anti-mouse IgG_1(γ1) (IgG_1-594), geneticin, fetal bovine serum (FBS), transferrin from human serum alexa fluor 594 conjugate, 4–12% bis-tris gels, invitrolon PVDF/filter paper, rprotein-G sepharose 4B conjugate beads, 20× MOPS SDS running buffer and 20× transfer buffer were purchased from Invitrogen (Naerum, Denmark). N-terminally SNAP-tagged human GLP-1R construct (SNAP-GLP-1R), SNAP-tagged β_2AR construct (SNAP-β_2AR), tag-lite® SNAP lumi4®-Tb (terbium energy donor), tag-lite internalization reagent (energy acceptor), and SNAP/CLIP lab medium 5× were purchased from Cisbio Bioassays (Codolet, France). LANCE/DELFIA D400 and CFP/YFP (D450_515) single mirrors, excitation filters X340_101 and CFP-430 (X430), emission filters 520/8, M615_203, CFP-470 (M470), and YFP-535 (M535), and sterile and tissue culture treated white opaque 96-well microplates were purchased from Perkin Elmer (USA). ECM gel from engelbreth-holm-swarm mouse sarcoma, poly-D-lysine hydribromide, tris base, $CaCl_2$, $MgCl_2$, HEPES, tween20, L-gluthathione reduced, iodoacetamide, albumin from bovine serum, Tris-Cl and isoproterenol were purchased from Sigma Aldrich (Broendby, Denmark). NaCl and KCl were purchased from Merck (Whitehouse Station, USA). NaOH and EZ-link sulfo-NHS-SS-biotin were purchased from Thermo Fisher Scientific (USA). Human embryonic kidney cells (HEK293) were purchased from ATTC (Boras, Sweden). C57BL6 mice were purchased from Taconic. SNAP-surface® alexa fluor® 488 (SNAP-substrate-488), PNGase F cocktail and rabbit polyclonal SNAP-tag antibody were purchased from New England Biolabs (Ipswich, USA). Paraformaldehyde (PFA) and OCT were purchased from VWR Bie & Berntsen (Herlev, Denmark). Vectorshield mounting medium with DAPI and Vectastain ABC Kit peroxidase standard PK-4000 were purchased from Vector Laboratories (Burlingame, USA). Faramount mounting media was purchased from DAKO. Lumigen PS3 detection reagent was purchased from GE Healthcare (USA). Sotalol was purchased from Tocris Bioscience (UK). 8-well permanox lab-tek® chamber slides™ were purchased from NUNC (Roskilde, Denmark). Black 384 tissue culture treated plates were purchased from BD Falcon (USA). Complete protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, USA). All unlabeled GLP-1R ligands were prepared in house using standard methods.

2.2. Synthesis of fluorescently labeled ligands

For visualization of GLP-1R ligands, GLP-1, exendin-4, exendin9-39 and liraglutide were synthetically labeled with red fluorophores or VivoTag750. N-alpha-Fmoc-[Arg26,Arg34,Lys37]-GLP-1 (GLP-1-synth) was synthesized on Fmoc-Lys-Wang resin whilst N-alpha-Fmoc-[Leu14,Arg27,Leu28]-exendin-4 (exendin-4-synth), [Leu14,Leu28]-exendin-4-Cys-amide (exendin-4C-synth), [Leu14,-Leu28]-exendin9-39-Cys-amide (exendin9-39-synth), and liraglutide-Cys-amide (liraglutide-synth) were synthesized on PAL or Rink ChemMatrix resin by standard Fmoc chemistry using a Liberty peptide synthesizer. After purification by RP-HPLC, 2 μmol of GLP-1-synth and exendin-4-synth were reacted with 1 mg Alexa594-NHS in 200 μl DMSO and 2 μl triethylamine for 2 h. Following, 4 μl of piperidine were added, and after 10 min the Fmoc protecting group was completely removed. For liraglutide, 2 μmol of liraglutide-synth was reacted with 1 mg Alexa594-maleimide in 200 μl DMSO and 2 μl collidine for 2 h. For exendin-4(VivoTag750) and exendin9-39(VivoTag750), 0.7 μmol of exendin-4C-synth or exendin9-39-synth were reacted with 1 mg VivoTag750-maleimide in 100 μl DMSO and 2 μl collidine for 30 min. N-epsilon37-Alexa594-[Arg26,Arg34,Lys37]-GLP-1 (referred to as GLP-1-594), N-epsilon12-Alexa594-[Leu14,Arg27,Leu28]-exendin-4 (referred to as exendin-4-594), and liraglutide-Cys(Alexa594)-amide (referred to as liraglutide-594) were purified by RP-HPLC and their theoretical masses of 4189, 4902, and 4742 Da, respectively, were confirmed by mass spectrometry. For exendin-4-cys(VivoTag750)-amide (referred to as exendin-4-VT750) and exendin9-39-cys(VivoTag750)-amide (referred to as exendin9-39-VT750), 200 μl 20 mM ammonium bicarbonate was added and the peptides purified using a PD-10 column eluted with 20 mM ammonium bicarbonate. The labeled peptides eluted first and were collected in 1.2 ml eluate and lyophilized. The fluorescent labeling of liraglutide does not alter the binding properties to GLP-1R, whilst the binding affinities of GLP-1-594 and exendin-4-594 change marginally compared to unlabeled ligands (Supplementary Fig. 1).

2.3. SNAP-tagging of GLP-1R

For visualization of GLP-1R, a SNAP-tag epitope was fused to the extracellular N-terminus of the human GLP-1R (Cisbio Bioassays, 2012). The SNAP-tag is a derivative of O6-guanine nucleotide alkyltransferase, which can covalently react with e.g. fluorescent-conjugated benzyl guanine substrates (Maurel et al., 2008). The N-terminal SNAP-tag on GLP-1R does not alter the binding properties of GLP-1 to the receptor (Supplementary Fig. 2).

2.4. Cell culture

To generate a cell line stably expressing the SNAP-GLP-1R HEK293 (referred to as SNAP-GLP-1R cells), HEK293 cells were transfected with the SNAP-GLP-1R plasmid using Lipofectamine2000. In addition, a HEK293 cell line stably expressing both the SNAP-GLP-1R and the EPAC[7] cAMP sensor with mCerulean and mCitrine as FRET pair as described previously (Mathiesen et al., 2013) was also generated (referred to as GLP-1R-EPAC cells). Cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and 0.8 mg/ml geneticin for selection of positive clones in a humidified 5% CO_2 air incubator at 37 °C.

2.5. Real Time cAMP signaling assay using an EPAC-sensor

GLP-1R-EPAC cells were seeded in sterile, black 384-well microplates coated with ECM and cultured overnight. Cells were

[6] Fluorescence Resonance Energy Transfer.

[7] Exchange protein activated by cAMP.

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