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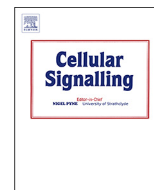

An incretin-based tri-agonist promotes superior insulin secretion from murine pancreatic islets via PLC activation

Noushafarin Khajavi[a,*], Brian Finan[b], Oliver Kluth[c], Timo D. Müller[b], Stefan Mergler[d], Angela Schulz[e], Gunnar Kleinau[a,f], Patrick Scheerer[f], Annette Schürmann[c], Thomas Gudermann[g,h,i], Matthias H. Tschöp[b], Heiko Krude[a], Richard D. DiMarchi[j], Heike Biebermann[a]

[a] *Institute of Experimental Pediatric Endocrinology, Charité - Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany*
[b] *Institute for Diabetes and Obesity, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH) and Technical University Munich, D-85748, Munich, Germany; German Center for Diabetes Research, D-85764, Neuherberg, Germany*
[c] *Department of Experimental Diabetology, German Institute of Human Nutrition Potsdam-Rehbruecke, Nuthetal, Germany; German Center for Diabetes Research, D-85764, Neuherberg, Germany*
[d] *Klinik für Augenheilkunde, Charité – Universitätsmedizin Berlin, Freie Universität Berlin, Humboldt-Universität zu Berlin, Berlin Institute of Health, Augustenburger Platz 1, 13353 Berlin, Germany*
[e] *Rudolf Schönheimer Institute of Biochemistry, Leipzig University, Leipzig, Germany*
[f] *Institute of Medical Physics and Biophysics, Group Protein X-ray Crystallography and Signal Transduction, Charité - Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin and Berlin Institute of Health, Berlin, Germany*
[g] *Walther Straub Institute of Pharmacology and Toxicology, LMU Munich, Munich, Germany*
[h] *German Center for Lung Research, Munich, Germany*
[i] *German Center for Cardiovascular Research, Munich Heart Alliance, Munich, Germany*
[j] *Department of Chemistry, Indiana University, Bloomington, IN 47405, USA*

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ABSTRACT

Recently, a unimolecular tri-agonist with activity at glucagon-like peptide 1 receptor (GLP-1R), glucose dependent insulinotropic receptor, and the glucagon receptor was reported to improve glycemic control in mice. Here, we defined the underlying molecular mechanisms of enhanced insulin secretion in murine pancreatic islets for a specific tri-agonist. The tri-agonist induced an increase in insulin secretion from murine islets compared to the respective mono-agonists. GLP-1R mainly signals via activation of the $G\alpha_s$ pathway, but inhibition of protein kinase A (H89) and exchange protein activation by cAMP (EPAC) (ESI-09) could not completely block insulin release induced by tri-agonist. Electrophysiological observations identified a strong increase of intracellular Ca^{2+} concentration and whole-cell currents induced by tri-agonist via transient receptor potential channels (TRPs). Although, EPAC activation mobilizes intracellular Ca^{2+} via TRPs, the TRPs blockers (La^{3+} and Ruthenium Red) had a larger inhibitory impact than ESI-09 on tri-agonist stimulatory effects. To test for other potential mechanisms, we blocked PLC activity (U73122) which reduced the superior effect of tri-agonist to induce insulin secretion, and partially inhibited the induced Ca^{2+} influx. This result suggests that the relative effect of tri-agonist on insulin secretion caused by GLP-1R agonism is mediated mainly via $G\alpha_s$ signaling and partially by activation of PLC. Therefore, the large portion of the increased intracellular Ca^{2+} concentration and the enhanced whole-cell currents induced by tri-agonist might be attributable to TRP channel activation resulting from signaling through multiple G-proteins. Here, we suggest that broadened intracellular signaling may account for the superior in vivo effects observed with tri-agonism.

Abbreviations: AC, Adenylyl cyclase; cAMP, Cyclic adenosine monophosphate; ECL, Extracellular loop; EPAC, Exchange protein directly activated by cAMP; FCS, Fetal calf serum; GcgR, Glucagon receptor; GIP, Glucose-dependent insulinotropic polypeptide; GIPR, Glucose-dependent insulinotropic polypeptide receptor; GLP-1, Glucagon-like peptide-1; GLP-1R, Glucagon-like peptide-1 receptor; GPCR, G-protein coupled receptor; HBSS, Hank's buffered salt solution; La^{3+}, Lanthanum-III-chloride; PKA, Protein kinase A; PKC, Protein kinase C; PLC, Phospholipase C; T2D, Type 2 diabetes; TMH, Transmembrane helix; TRPs, Transient receptor potential channels; TRPM, Transient receptor potential melastatin; VDCCs, Voltage-dependent Ca^{2+} channels

* Corresponding author at: Institut für Experimentelle Pädiatrische Endokrinologie, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany.
E-mail address: Noushafarin.khajavi@charite.de (N. Khajavi).

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1. Introduction

G protein-coupled receptors (GPCRs) are essential elements in the regulation of physiological processes and as such constitute high priority targets for drug discovery [1]. Incretin receptors are class B GPCRs [2] and targeting these receptors is a proven approach to manage type 2 diabetes (T2D), and lower body weight [3, 4]. Up to 60% of the insulin secretory response to food consumption is due to the insulinotropic effects of incretins and especially glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) [5]. Due to the essential role of incretins in mediating insulin secretion, therapeutic strategies based on activating GLP-1R and GIPR on β-cells are being developed [6, 7]. To potentiate the therapeutic effect, simultaneous modulation of these incretin receptors, either by co-administration of individual agonists or by unimolecular multi-agonists, is being advanced as novel treatments of obesity and T2D [8–12]. The pharmacological rationale relates to addition of the thermogenic and lipolytic activities of glucagon, with the insulinotropic and insulin sensitizing properties of GIP to well-established GLP-1 based biology [10, 13]. As a proof of concept, multi-agonist administration to obese mice was shown to improve their metabolic dysfunction more than that achieved by the respective mono-agonists [10]. However, the underlying molecular mechanism that results in greater efficacy with multi-agonist treatment relative to mono-agonists or co-administration of several compounds remains undefined. A plausible approach to address this question is the study of the molecular mechanism of insulin secretion induced by the tri-agonist in pancreatic islets. The cells are a primary target for incretin action, and incretin receptors as well as the glucagon receptor are known to be functionally present.

It is well established that GLP-1R and GIPR signal via activation of the $G\alpha_s$/adenylyl cyclase (AC) pathway in pancreatic β-cells [14–16], which leads to activation of protein kinase A (PKA) and exchange protein activation by cAMP (EPAC). In high glucose condition, PKA activation facilitates the closure of ATP-sensitive K^+ channels (K_{ATP} channels), membrane depolarization and influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCCs). The subsequent increases of $[Ca^{2+}]_i$ triggers insulin exocytosis [15, 17, 18]. There is mounting evidence that GLP-1R is capable of signaling through additional G-protein subtypes, such as $G\alpha_{i/o}$ and $G\alpha_{q/11}$ proteins [19, 20]. Activation of $G\alpha_{q/11}$ results in downstream signaling through phospholipase C (PLC) and subsequently protein kinase C (PKC) activation. Nonetheless, several studies have demonstrated that cAMP is the main mediator of GLP-1 action on acute molecular events associated with insulin secretion [18, 21, 22].

K_{ATP} channels are known to act downstream of G-protein activation and their closure is crucial for insulin exocytosis in pancreatic β-cells. However, electrophysiological studies have demonstrated that it is not sufficient to shift the membrane potential towards a threshold level to suggest that additional ion channels such as transient receptor potential channels (TRPs) might facilitate depolarization after K_{ATP} channel closure [23–25]. It has also been reported that GLP-1 is capable of depolarizing β cells in the presence of a VDCCs blocker, implying the involvement of other cation-conducting channels [25]. Recent studies demonstrated that GLP-1 receptor stimulation induces oscillations of cAMP which potentiate $[Ca^{2+}]_i$ transient amplification through activation of the cAMP/EPAC/transient receptor potential melastatin 2 (TRPM2) signaling pathway [26, 27]. Moreover, PKC activation mediates membrane depolarization due to activation of Na^+-permeable TRPM4 and TRPM5 channels. Finally, increased action potential firing rates lead to Ca^{2+} influx and stimulation of insulin exocytosis [18, 25]. In this study we observed an elaborate G-protein signaling network and subsequent ion channel activation that results in down-stream signal amplification induced by a specific tri-agonist. These results may explain the additional therapeutic benefit inherent to simultaneous cellular activation through multiple signaling pathways.

2. Materials and methods

2.1. Isolation of mouse islets and determination of insulin secretion

Isolation of islets was performed by a modified protocol of Gotoh et al. (1990) from C57BL/6 mice [28]. The pancreas was perfused by injection of 3 mM Collagenase-P (Roche, Mannheim, Germany) (0.3 mg/ml) in Hank's buffered salt solution (HBSS) containing 25 mM HEPES and 0.5% (w/v) BSA into the common bile duct. Isolated islets were recovered for 2 days in RPMI 1640 (PAA, Laborbedarf, Austria) in humidified 5% CO_2, 95% air at 37 °C.

Before determination of insulin secretion, islets were equilibrated for 1 h in KRBH-Buffer (115 mM NaCl, 4.5 mM KCl, 1.2 mM KH_2PO_4, 2.6 mM $CaCl_2$, 10 mM HEPES, 20 mM $NaHCO_3$, 0.2% (w/v) BSA, pH 7.4) with 2.8 mM glucose. Determination of insulin secretion from the islets was performed in 24-well plates containing 300 µl KRBH (10 islets/well, 3–4 independent experiments performed in duplicate). First, islets were incubated for 1 h in KRBH with 2.8 mM glucose followed by 1 h incubation in 20 mM glucose both supplemented with mono- or multi-agonist. 10 µM H89 (PKA inhibitor), 10 µM ESI-09 (EPAC inhibitor), 10 µM nifedipine (VDCCs blocker), 100 µM lanthanum-III-chloride (La^{3+}) (TRPs blocker) and 1 µM U73122 (PLC blocker) (Sigma-Aldrich, Taufkirchen, Germany) were used as specific inhibitors. These specific blockers of signaling were applied in concentrations of maximum inhibitory strength [29–34]. Islets were incubated in the presence of mono- or multi-agonists, all at a maximum stimulatory concentration, with or without aforementioned inhibitors. Released insulin was measured in the supernatant using an insulin ELISA kit (ALPCO, Salem, US) and normalized to the DNA content of the islets as determined by an DNA assay kit (Quant-iTPicoGreen dsDNA Assay Kit, Thermo Fisher Scientific, Waltham, US).

2.2. Cell culture

1.1B4 cells (European collection of cell cultures, UK) were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37 °C.

2.3. Determination of $G\alpha_s$/adenylyl cyclase activation by alpha screen technology

For determination of $G\alpha_s$ signaling, cAMP accumulation in 1.1B4 cells was measured. Cells were stimulated for 45 min with GLP-1 or tri-agonist (decade concentration response curves starting from 1 µM) in stimulation buffer containing 20 mM glucose (high glucose condition) and 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich, St. Louis, MO). Accumulation of cAMP was determined by AlphaScreen technology (Perkin Elmer, Life Science, Zaventem, Belgium). Stimulation and measurements of down-stream signaling were performed as previously described [35].

2.4. Determination of intracellular Ca^{2+} concentration

To monitor time-dependent changes in intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in single-cells, fura-2 fluorescence measurements were performed. In brief, the 1.1B4 cells were cultivated on 15 mm diameter glass cover slips placed in a culture plate until they reached a semi confluent stage (≈70%). Cells were pre-incubated with 2 µM fura-2/AM for 30 min, at 37 °C. Loading was stopped with the extracellular Ringer-like solution contained: 150 mM NaCl, 6 mM CsCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES acid and 20 mM glucose (pH of 7.4 and osmolality of 320 mOsM). After rinsing the cells with this solution, fluorescence measurements were performed for 250–450 s at room temperature (21–23 °C) on the stage of an inverted microscope (Olympus BW50WI) and a camera (Olympus XM-10) in connection with a LED-Hub (Omikron, Rodgau-Dudenhoven, Germany). Fura-2

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