The regulatory G protein signaling complex, Gβ5-R7, promotes glucose- and extracellular signal stimulated insulin secretion

Qiang Wang ‡†, Taylor A. N. Henry ‡†, Alexey N. Pronin ‡, Geeng-Fu Jang §, Camila Lubaczeuski¶, John W. Crabb §, Ernesto Bernal-Mizrachi ¶, and Vladlen Z. Slepak ‡*

From the ‡‡ Department of Molecular and Cellular Pharmacology and ¶ Division of Endocrinology, Diabetes and Metabolism, University of Miami School of Medicine, Miami, Florida, 33136 and § Cole Eye Institute & Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, 44195

Running Title: Gβ5-R7 facilitates glucose-stimulated insulin secretion

*To whom correspondence should be addressed: University of Miami School of Medicine, R-189, 1600 NW 10th Ave., Miami, FL 33136. Tel:305-243-3430. Fax: 305-243-4555. E-mail: vslepak@med.miami.edu.

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ABSTRACT

G protein-coupled receptors (GPCRs) are important modulators of glucose-stimulated insulin secretion (GSIS), essential for maintaining energy homeostasis. Here, we investigated the role of Gβ5-R7, a protein complex consisting of the atypical G protein β subunit Gβ5 and a regulator of G protein signaling (RGS) of the R7 family. Using the mouse insulinoma MIN6 cell line and pancreatic islets, we investigated the effects of G protein subunit 5 (Gnb5) knockout on insulin secretion. Consistent with previous work, the Gnb5 knockout diminished insulin secretion evoked by the muscarinic cholinergic agonist Oxo-M. We found that the Gnb5 knockout also attenuated activities of other GPCR agonists, including ADP, arginine vasopressin (AVP), glucagon-like peptide 1 (GLP-1), and forskolin, and surprisingly, the response to high glucose. Experiments with MIN6 cells cultured at different densities provided evidence that the Gnb5 knockout eliminated the stimulatory effect of cell adhesion on Oxo-M stimulated GSIS; this effect likely involved the adhesion GPCR GPR56. The Gnb5 knockout did not influence cortical actin depolymerization, but affected protein kinase C activity, and the 14-3-3ε substrate. Importantly, Gnb5 -/- islets or MIN6 cells had normal total insulin content and released normal insulin amounts in response to K+-evoked membrane depolarization. These results indicate that Gβ5-R7 plays a role in the insulin secretory pathway downstream of signaling via all GPCRs and glucose. We propose that the Gβ5-R7 complex regulates a phosphorylation event participating in the vesicular trafficking pathway, downstream of G protein signaling and actin depolymerization, but upstream of insulin granule release.

Maintaining the appropriate concentration of blood glucose is one of the most crucial homeostatic functions of the body. Glucose levels rise after ingestion of food or when activation of the sympathetic nervous system stimulates the release of glucose from its storage in the liver and skeletal muscle. Glucose levels return to normal once the demand for energy subsides and tissues metabolize or store excess glucose. Disruption of this delicate balance results in the development of diseases such as diabetes. Glucose uptake is stimulated by insulin, the hormone synthesized and released by a single cell type in the body: the beta cells located in the pancreatic islets.

The basic mechanism of glucose-stimulated insulin secretion (GSIS) was proposed...
more than two decades ago and is as follows. Upon the rise of blood glucose, its enhanced transport into the beta cells boosts production of ATP, causing closing of the ATP-sensitive K⁺ channels and depolarization of the plasma membrane. Depolarization promotes opening of L-type voltage-gated Ca²⁺ channels and the influx of extracellular Ca²⁺ into the cytosol, triggering the exocytosis of insulin-containing vesicles. This model is therefore referred to as the triggering pathway, as glucose transport into the cell triggers this increase in cytosolic Ca²⁺ (1,2).

A more complete model of insulin secretion incorporates the biphasic nature of secretion, cytoskeletal remodeling, and the transport and docking of insulin granules on the plasma membrane, all of which are regulated by signaling mechanisms (3-5).

Insulin release is suppressed in the absence of high glucose, as excess uptake leads to hypoglycemia. However, at permissive glucose levels, beta cells are responsive to a multitude of hormones, neurotransmitters and other extracellular stimuli that enhance or attenuate GSIS. These signals operate via the metabolic amplification pathway, which is thought to operate during the second phase of GSIS, when insulin secretion is limited to fine-tuned pulses, released as necessary (1,2). Many of GSIS-modulating inputs are mediated by G protein-coupled receptors (GPCRs). For example, receptors of glucagon-like peptide GLP-1 promote GSIS via activation of Gₛ and the corresponding rise in cAMP (6,7). Receptors of vasopressin and adenosine promote GSIS via activation of Gₛ (8-10). Cholinergic stimulation, which in the beta cells is integrated via the Gₛ-coupled muscarinic cholinergic receptor, M3 (M3R), also has a strong insulinotropic effect (11,12).

G protein signaling involves a number of regulatory proteins, including arrestins and protein kinases that regulate the functions of GPCRs and the downstream events. Regulator of G protein signaling (RGS) proteins are a diverse family characterized by the presence of the ~100 amino acid RGS domain that interacts with GTP-bound Gₛ subunits and accelerates their GTPase activity (13,14). While the GAP activity is a hallmark function of RGS proteins, many of them have additional domains that perform other functions. RGS proteins that belong to the R7 family (RGS6, RGS7, RGS9 and RGS11) form obligate heterodimers with Gβ5, an atypical Gβ subunit (15). The R7 RGS subunit consists of four domains: RGS, GGL (G gamma-like), DHEX and DEP (16). Gβ5 binds to the GGL domain, and this interaction is obligatory: they have never been found separately in vivo, and the Gβ5 and R7 subunits quickly degrade if expressed separately in vitro (17). Therefore, the knockout of Gnb5 causes ablation of the entire R7 family (18). In addition to Gₛ, the Gβ5-R7 dimers interact with anchoring proteins, ion channels, GPCRs, and other molecules (19,20). According to a recent report, Gβ5-RGS7 can directly interact with G₁₂/₁₃, influencing the activity of the Rho pathway and cytoskeletal rearrangement in neuronal cells (21).

Gβ5-R7 complexes are highly expressed in the nervous system, and were originally referred to as neuronal proteins. Subsequent studies showed their presence at a lower level in other tissues and cell types (22-26). In contrast to investigations in the nervous system, the role of RGS proteins in pancreas has been relatively unexplored. It was shown that pancreatic expression of RGS16 and RGS8 is low in normal adults and high in those with diabetes (27). Another study investigated RGS4 and demonstrated that it acts as a negative regulator of insulin secretion stimulated by M3R and other receptors (28). Surprisingly, our earlier studies showed that Gβ5-R7 acted as a positive regulator of M3R-stimulated insulin secretion. The Gnb5 knockout in mice dramatically reduced serum insulin levels, and these findings were consistent with the CRISPR/Cas9-mediated Gnb5 knockout and overexpression in MIN6 cells (24,29). In this paper, we extended the studies of Gβ5-R7 in beta cells and our results indicate that Gβ5-R7 enhances not only the function of M3R, but also that of other stimuli including the insulinotropic activity of high glucose.

Results

Our previous work demonstrated that the Gnb5 knockout caused a dramatic reduction of M3R-stimulated GSIS in MIN6 cells (24), and in
this study we further investigate the cellular and molecular mechanisms affected by the knockout.

**Gnb5 knockout does not impair actin depolymerization**

GSIS is a biphasic process that is characterized by a rapid first phase and a slower, continuous second phase. During the first phase, beta cells release insulin granules that are pre-docked on the plasma membrane. In the second phase, insulin-containing vesicles are recruited from intracellular storage pools. A key step in this second phase is local depolymerization of the cortical F-actin filaments that block the passage of the vesicles to the plasma membrane until the arrival of the appropriate signal(s) (30,31). One of the regulators of cortical F-actin dynamics is Rho-associated protein kinase (ROCK), and Gβ5-RGS7 complex was implicated in regulation of actin cytoskeleton via G13 and RhoGEF proteins in neurons (21,32). Therefore, we hypothesized that the knockout of Gβ5 may disrupt actin remodeling in MIN6 cells. If depolymerization were impaired in Gnb5-/- cells, we could expect that application of latrunculin B, an inhibitor of actin polymerization, would rescue insulin secretion.

The effect of latrunculin in 3 mM glucose was minimal (Fig 1A), which was predictable, as insulin secretion is repressed in low glucose. Nevertheless, in the Gnb5 +/- cells, stimulation with muscarinic agonist Oxo-M resulted in a 2-fold increase (from 0.65 ± 0.12 μg/L to 1.3 ± 0.26 μg/L n=5, p=0.00198) in insulin secretion. Application of latrunculin resulted in an almost identical stimulation of insulin release. In the presence of high (16.7 mM) glucose latrunculin also stimulated insulin release approximately 2-fold (Fig 1B). However, the overall amount of latrunculin-stimulated insulin release in high glucose was 3 times larger than in low glucose (3.71 ± 1.14 in 16.7 mM vs. 1.37 ± 0.27 μg/L in 3 mM, n=5, p=0.004) (Fig 1B). Consistent with previous studies, Oxo-M stimulated GSIS 4-fold in the Gnb5 +/- cells, and this response was slightly potentiated by latrunculin.

The Gnb5 knockout markedly suppressed insulin secretion (Fig 1), consistent with our finding that Gβ5-R7 is a positive regulator of GSIS (24). In low glucose, Oxo-M caused a statistically significant increase in secretion (from 0.46 ± 0.01 μg/L to 0.77 ± 0.33 μg/L, n=5, p=0.005), but this amount was 2 times lower than the amount secreted in Gnb5 +/- cells. In high glucose, Gnb5 +/- cells secrete about 6 times less insulin with Oxo-M stimulation than the control cells. Latrunculin did not have an effect on secretion in low glucose, but in high glucose it caused a slightly stronger stimulation than Oxo-M (0.77 ± 0.16 μg/L vs. 0.93 ± 0.08 μg/L, n=5, p=0.34). Application of latrunculin together with Oxo-M boosted secretion to 1.5 ± 0.44 μg/L. However, this amount was still underwhelming in comparison to the Gnb5 +/- cells (Fig 1B).

Together, these results show that latrunculin facilitates insulin secretion regardless of the presence of Gβ5-R7. Oxo-M could not stimulate the Gnb5-/- cells to secrete insulin to a degree comparable to that of Gnb5 +/- even when latrunculin caused disassembly of cortical actin barrier. Accordingly, when we investigated effects of ROCK and Rho inhibitors on Oxo-M-stimulated insulin secretion (data not shown), we did not find any evidence of a link between Gβ5-RGS7 and regulation of insulin secretion by the Rho/ROCK pathway. We conclude from these experiments that Gβ5-R7 does not play a role in the actin depolymerization step of insulin exocytosis.

Our results also revealed that, with or without latrunculin, the effect of high glucose is diminished in the Gnb5-/- cells.

**Gnb5 knockout reduces GSIS in MIN6 cells and primary pancreatic islets**

MIN6 cells are known to have relatively low GSIS compared to isolated pancreatic islets. Therefore, in our earlier studies of the role of Gβ5-R7 in MIN6 cells we focused on muscarinic stimulation of GSIS via M3R, which has a broad dynamic range (24). However, the amplification of insulin responses by latrunculin (Fig 1) made the effect of Gnb5 knockout on GSIS obvious (Fig 2A). In Gnb5 +/- MIN6 cells, the increase in glucose concentration from 3.3 mM to 16.7 mM induces an almost 2-fold rise in insulin secretion (from 0.65 ± 0.12 μg/L to 1.13 ± 0.11 μg/L, n=5, p=0.0004). This increase is modest in comparison to the responses to Oxo-M (Fig 1), but is statistically significant. In contrast, high glucose did not significantly stimulate the Gnb5-/- cells.
(from 0.46 ± 0.1 to 0.48 ± 0.16 μg/L, n=5, p=0.66).

Importantly, we observed a similar effect of Gnb5 knockout on GSIS using pancreatic islets isolated from wild-type and Gnb5 knockout mice (Fig 2B). In control islets, application of 16.7 mM glucose stimulated insulin secretion was increased almost 10-fold (from 0.36 ± 0.05 to 3.54 ± 1 μg/L; n=3, p=0.003). In the Gnb5-/- islets, this stimulation was only 4-fold (from 0.36 ± 0.04 to 1.44 ± 0.72 μg/L n=5, p=0.07) (Fig 2B).

Membrane depolarization, total insulin content and stimulation of GPCRs in MIN6 cells and islets

We tested if the Gnb5 knockout affected the responses to stimuli other than glucose and M3R agonist Oxo-M (Fig 3). Treatment of Gnb5+/+ and Gnb5-/- MIN6 cells with 50 mM KCl evoked a similar response. Furthermore, there was no significant difference in the total insulin content, which was determined after complete lysis of the Gnb5-/- and Gnb5+/+ MIN6 cells. These results indicate that insulin production and cellular response to depolarization are not affected by the Gnb5 knockout. Similarly, there was no significant difference in KCl-evoked insulin release in pancreatic islets isolated from Gnb5+/+ and Gnb5-/- mice (Fig 3C). This is consistent with our previous data, which showed that total insulin content in control and knockout islets were indistinguishable (29).

Next, we tested how Gnb5-/- MIN6 cells respond to stimulation of GPCRs other than M3R (Fig 3B). Our data show that ADP and AVP, which are known to promote insulinotropic activity via Gq-coupled adenosine and vasopressin receptors, respectively, stimulated GSIS in the Gnb5 +/+ MIN6 cells. ADP in the presence of 16.7 mM glucose raised the insulin response from 1.82 μg/L to 3.37 μg/L, and AVP up to 4.31 μg/L. In the Gnb5 -/- cells, the ADP and AVP responses were essentially undetectable.

In Gnb5 +/+ cells, activation of the Gq-coupled receptor of glucagon-like peptide GLP-1 increased insulin release about 2-fold in comparison to high glucose alone. Direct activation of adenylate cyclase by forskolin had a similar effect. The relatively modest insulinotropic activity of the cAMP pathway is consistent with a report that GLP-1 receptor signaling is reduced in beta cells that have been exposed to chronic high glucose environments, as is the case with cultured MIN6 cells (33). In Gnb5 -/- cells, GLP-1 evoked a statistically significant response (1.55 ± 0.55 μg/L vs. 0.79 ± 0.27 μg/L; n=5, p=0.048); this increase was reduced compared to that of Gnb5 +/+ cells (1.55 ± 0.55 μg/L vs. 2.66 ± 0.66 μg/L; n=5, p=0.016). Similar results were obtained with forskolin, however the forskolin-stimulated increase in insulin secretion from the Gnb5 -/- cells was not statistically significant (1.18 ± 0.37 μg/L vs. 0.79 ± 0.27 μg/L; n=6; p=0.118), and the amount of insulin released in the presence of forskolin was less than half of the response of the Gnb5 +/+ cells (1.18 ± 0.36 μg/L vs. 2.8 ± 0.48 μg/L; n=6, p=1.38 x 10^-5).

In the primary pancreatic islets from Gnb5 +/+ mice (Fig 3D), Oxo-M stimulation resulted in a two-fold increase in insulin release (4.16 ± 0.87 vs. 8.99 ± 1.73 μg/L; n=3; p=0.00023) in high glucose, consistent with our previous report (29). Application of forskolin almost doubled the insulin response in these islets (4.16 ± 0.87 vs. 7.64 ± 2.5; n=3; p=0.01); in fact, there was no statistical difference between the responses evoked by Oxo-M and forskolin (p=0.34) (Fig 3B). Forskolin also potentiated insulin secretion by the Gnb5 -/- islets in the presence of high glucose (2.3 ± 0.37 vs. 4.2 ± 2.1; n=3; p=0.07), but the level of forskolin-evoked insulin release was 1.76 times lower than in the control islets.

These results show that the Gnb5 knockout (Fig 2) diminishes insulinotropic activity of not only M3R, but also that of a broader range of insulinotropic stimuli.

Cell Adhesion Signaling

In the course of our studies we noticed that M3R-mediated stimulation of insulin secretion was more robust when MIN6 cells were plated at higher densities. MIN6 cells are known to form aggregates, which show improved GSIS compared to less confluent cultures (34). Since total insulin content per cell is similar in large and small aggregates (35), it is thought that increased cell-to-cell communication facilitates secretion rather than production of insulin.
We investigated whether the loss of Gβ5-R7 affects this cell adhesion-related phenomenon. We plated Gnb5-/- and Gnb5+/+ MIN6 cells at densities ranging from 3x10^3 to 12x10^5 cells per well on 12-well plates (Fig 4). After 24 hours, both Gnb5-/- and Gnb5+/+ clones formed larger clusters when seeded at high density (Fig 4A). Analysis by western blot (Fig 4B) and immunohistochemistry showed that Gnb5 knockout does not influence E-cadherin expression level; the same results were obtained when analyzing β-catenin and Connexin 36 (data not shown). These results indicate that the Gnb5 knockout does not affect cadherin-mediated adhesion signaling in MIN6 cells.

We then measured the Oxo-M-stimulated insulin release in these cultures. The values of insulin released (Fig 4C) in each well were normalized to the number of cells (Fig 4D).

For Gnb5+/+ cells, quadrupling the seeding density caused a 10-fold rise in insulin secretion per well, showing that the increase in insulin release is not directly proportional to that of cell number. Insulin secretion per cell rises from 4.13 ± 0.64 pg/L to 8.58 ± 1.33 pg/L (n=6, p=5.84 x10^-6), supporting the notion that signaling associated with beta cell adhesion improves stimulated insulin release (34,36). In the Gnb5-/- cells, quadrupling the plating density resulted in the proportional 4-fold increase in the amount of insulin release per well (from 0.50 ± 0.02 μg/L to 2.07 ± 0.57 μg/L), showing that individual cells release the same amount of insulin regardless of aggregation. We concluded that Gnb5 knockout prevents the improvement in secretory performance that occurs in Gnb5+/+ cells upon the increase of cell density.

To rule out the possibility that the enhancement in insulin secretion in larger MIN6 aggregates could be caused by soluble factors, we collected conditioned medium from high-density cultures and applied it to low-density cultures. This medium had no effect (data not shown). This finding implicates direct cell-to-cell contacts rather than a soluble factor(s) in promotion of GSIS in the denser MIN6 cultures; this is consistent with previous reports (35,37).

An interesting family of receptors mediating cell-to-cell adhesion and interactions with the extracellular matrix is adhesion GPCRs (38,39). The activity of adhesion GPCR GPR56 has been recently implicated in potentiating GSIS in beta cells (40). A known endogenous ligand of GPR56 is collagen III, which promotes signaling through G13 and Rho pathway (38). GPR56 is also activated by P7, a synthetic peptide designed to mimic the intrinsic agonist sequence of the receptor (39). We compared the response to these two ligands in our Gnb5+/+ and Gnb5-/- MIN6 cells (Fig 5). In Gnb5+/+ cells, treatment with 0.5 μM collagen III resulted in an almost 2-fold (from 0.99 ± 0.07 μg/L to 1.78 ± 0.62 μg/L) enhancement of GSIS in comparison to samples treated with 16.7 mM glucose only. This stimulation was lower than with Oxo-M, but is similar to values obtained with such secretagogues as GLP-1 and forskolin (Fig 3). Treatment of Gnb5+/+ cells with 50 μM P7 resulted in a similar stimulation as treatment with collagen III (1.54 ± 0.33 μg/L). The Gnb5 knockout abrogated the effects of both ligands, which is consistent with the idea that Gβ5-R7 in MIN6 cells promotes multiple insulinotropic pathways, including G12/13.

**Effect of Gnb5 knockout on PKC-mediated phosphorylation**

Our earlier study indicated that Gβ5-R7 may regulate Oxo-M-mediated insulin release via changes in protein kinase activity (24). Here, we probed MIN6 cell lysates with an antibody raised against a phosphorylated peptide corresponding to the PKC substrate consensus sequence – (K/R)XpSX(K/R). According to the immunoblot analysis, treatment of MIN6 cells with Oxo-M resulted in a notable increase in phosphorylation of multiple proteins (Fig 6A). Overall patterns of PKC-mediated phosphorylation were very similar between the Gnb5 +/+ and Gnb5 -/- MIN6 clones. However, phosphorylation of one of the proteins with the apparent molecular mass ~28 kDa (p28) was increased about 2-fold in the Gnb5-/- cells.

To identify p28 we excised the protein band from the gel and performed mass spectrometry analysis. As expected, the gel slice contained hundreds of proteins. To narrow down the list, we took advantage of the fact that the mobility of proteins slightly changes depending on the buffer system used in electrophoresis.
Indeed, when resolved on a bis-Tris gel using MES running buffer, p28 moved slower relative to 25 and 37 kDa protein standards (Fig 6B), with an apparent molecular mass of ~30 kDa. We expected proteins co-migrating with p28 in the “30 kDa” band to be different from those that co-migrated with p28 in the Tris-glycine system (Fig 6C). To distinguish p28 from contaminants, we searched for proteins that were enriched in the ~28 kDa area of the gel (highlighted in red, Fig 6C) compared to the adjacent gel slices (green and blue); more than 20 proteins fit that criterion. Analysis of all sets of mass-spectrometry data showed that most proteins enriched in the “p28” (Tris-glycine) and 30 kDa (Tris/MES) bands were different. However, one protein was at the top of the list in both datasets: 14-3-3ε. We confirmed this identity using western blot analysis with a mixture of the phospho-PKC substrate (green) and 14-3-3ε (red) antibodies (Fig 6D). Furthermore, the most abundant phosphopeptide identified via the mass spectrometry analysis was the 14-3-3ε peptide containing Ser213, which we believe to be the one phosphorylated by PKC. (Fig 6E).

Discussion

The endocrine pancreas responds to fluctuations of the plasma glucose concentration and to a variety of other cues. For example, the nervous system can prime the pancreas to upcoming nutrient intake, enhancing GSIS via released acetylcholine (12,28,41). Multiple membrane receptors on beta cells augment or suppress GSIS, and the network of downstream signaling proteins integrates the stimuli and optimizes the resulting insulin output. Our previous study showed that ablation of the Gβ5-R7 complex caused a reduction of the serum insulin level in vivo (29), and subsequent experiments on isolated islets and MIN6 cells supported the notion that Gβ5-R7 is a positive modulator of cholinergic stimulation of GSIS (24). Our analyses of Oxo-M-stimulated fluxes of Ca2+, cAMP and DAG did not reveal a notable change in Gnb5−/− MIN6 cells, and in Gnb5−/− islets there was only a small reduction in the frequency of Ca2+ oscillations (24). The apparent promotion of M3R-stimulated GSIS by Gβ5-R7 was unexpected because RGS proteins are known as inhibitors of GPCR signaling. In this paper we further explored the role of Gβ5-R7 in insulin secretion.

The main finding reported here is that the knockout of Gnb5 suppresses insulin release stimulated not only by cholinergic receptor M3R but also by other GPCRs and even glucose (Figs 2, 3, 5). In our previous studies, we concentrated on the role of Gβ5-R7 in regulation of M3R-mediated insulin secretion rather than other insulinotropic pathways because muscarinic agonists are 3-6 times more efficacious than other secretagogues (Fig 3, (11,24)). In this study, our experiments with latrunculin B enhanced all tested MIN6 responses (Fig 1), highlighting the negative effects of Gnb5 knockout on the insulinotropic effects of glucose (Fig 2). The subsequent experiments provided evidence that the Gβ5-R7 complex is also needed for appropriate signaling through other Gq, G12, and G13-coupled receptors. At the same time, our data on both MIN6 cells and islets showed that total insulin content or its release caused by K+-induced membrane depolarization were indistinguishable between the Gnb5−/− and Gnb5+/+ phenotypes. Thus, the reduction in insulin release by Gnb5−/− MIN6 cells and primary islets cannot be attributed to changes in insulin content or membrane potential and suggests that Gβ5-R7 has a role in controlling the metabolic amplifying pathway (1,2).

Another interesting finding made in this paper concerns GPR56 (gene: ADGRG1), an adhesion GPCR that is highly expressed in beta cells (42). GPR56 couples to G12/13 and is activated by collagen III and the seven amino acid fragment (P7) of the extracellular domain, which acts as a tethered agonist (39). Our data showed that both collagen III and P7 facilitated GSIS in Gnb5+/+, but not in Gnb5−/− cells (Fig 5), indicating that Gβ5-R7 is required for GPR56 and/or G12-mediated signaling. One possible mechanism explaining this effect could be the involvement of the direct interaction between Gβ5-R7 and G12/13. It was recently reported that Gβ5-RGS7 complex co-immunoprecipitated with G13 from neuroblastoma cell line Neuro2A, and this interaction was implicated in Rho signaling and actin dynamics (21). Although in this study we did not find an obvious link between Gβ5-R7
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and the Rho pathway and/or actin remodeling, the regulation of G₁₂/₁₃ signaling by the Gβ5-R7 complex warrants further investigation.

While the Gβ5-R7 complex acts as an enhancer of insulinotropic stimuli in beta cells, it has an inhibitory effect in other biological systems. In various CNS neurons it has the canonical inhibitory role of an RGS protein, i.e., the knockouts of Gnb5 or R7 proteins enhanced Gᵢ signaling, evidently by extending the GTP-bound state of the G proteins (43). We also found that Gβ5-R7 attenuates cell functions in other systems, i.e., the Gnb5 knockout enhanced constriction of mouse pupillary smooth muscle via endogenous M₃R (24). In transfected CHO-K1 cells, Gβ5-RGS7 suppressed Ca²⁺ signaling induced by M₃R via a non-GAP mechanism that implies direct interaction with the receptor (44). Therefore, we propose that Gβ5-R7 promotes insulin secretion via a novel molecular mechanism, which needs to be understood.

The Gnb5 knockout hinders the effect of high glucose, a permissive factor for insulin secretion, which can explain why many GPCR pathways that modulate GSIS are affected. The breadth of the Gnb5 knockout effect on insulinotropic stimuli suggests that the mechanism promoted by Gβ5-R7 is situated downstream of multiple pathways. All secretory pathways converge to enhance exocytosis, a process that includes vesicular trafficking and membrane fusion. Our data showed that actin depolymerization does not require Gβ5-R7 (Fig 1), and so the Gβ5-R7-dependent insulinotropic event(s) is likely to be downstream of actin remodeling. Since the effect of K⁺-induced plasma membrane depolarization is not affected by the Gnb5 knockout (Fig 3), this step should be upstream of vesicle-membrane fusion.

Exocytosis is dependent on the formation of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex. This complex is composed of three main components: the vesicle-bound VAMP2, which binds to two target membrane proteins, syntaxin1A and SNAP25 (45). It was shown that SNAP25 in neuronal cells can directly interact with the conventional Gβγ complexes, influencing neurotransmitter release (46-48). Considering that the Gβ5-GGL moiety of the Gβ5-R7 complex may have a similar role as conventional Gβγ complexes, we hypothesize that Gβ5-R7 can promote the docking of insulin granules, i.e., increasing the pool of secretory vesicles that are ready for release. Furthermore, there is a structural homology between R7BP and syntaxin family SNARE complex proteins (49), providing a basis for a potential protein-protein interaction between the DEP domain of the R7 protein and syntaxin.

In beta cells, components of the SNARE complex, SNAP25, munc18, and synaptotagmin, have been identified as key substrates of PKC phosphorylation. Phosphorylation of these proteins is thought to promote insulin exocytosis through facilitating the formation of the SNARE complex, sensitizing the complex to calcium, and increasing the amount of primed insulin vesicles available for exocytosis (45). We hypothesize that Gβ5-R7 is required for regulation of exocytosis-related kinase activity, for example, serving as an adapter protein that facilitates phosphorylation of these substrates.

Our results show that Gnb5 knockout in beta cells affects phosphorylation patterns evoked by Oxo-M stimulation, and we have begun identification of the substrates of PKC for which phosphorylation is dependent on the presence Gβ5-R7. So far, we have demonstrated that Gnb5 knockout enhances the phosphorylation of 14-3-3ε. The exact effect of this phosphorylation is not known, but it was shown that 14-3-3 proteins can stimulate exocytosis (50,51). Phosphorylation of 14-3-3 typically inhibits their interaction with target proteins (52). Therefore, we can speculate that by inhibiting 14-3-3ε phosphorylation, Gβ5-R7 promotes its interaction with the target proteins and, thus, exocytosis.

However, causal relationship between the Gnb5-dependent kinase activity and the effect of Gβ5-R7 on insulin secretion remains to be elucidated.
Experimental Procedures

Materials
Latrunculin B (ab144291) was purchased from Abcam (Cambridge, MA, USA). Oxotremorine M (Oxo-M, sc-203656) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against phospho-Ser PKC substrates (#2261) and polyclonal rabbit anti-E-cadherin (#3195) were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibody against actin (MAB1501R) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen III, forskolin, AVP, ADP and GLP-1 were also purchased from Sigma-Aldrich. Antibody against 14-3-3ε (sc-23957) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Tethered agonist peptide of GPR56, P7 (TYFAVLM) peptide (39) was kindly provided by Dr. Tall (University of Michigan). Cell culture inserts (PIXP01250) used in static islet experiments had a diameter of 12 mm and 12 µm pores and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals
Animal procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA) and protocols were approved by the University of Miami Committee on Use and Care of Animals. For the present study, Gβ5-knockout (Gbn5-/-) mice (18,24,29) were back-crossed for several generations to a C57Bl6/6J background. Age-matched (12-18 weeks old) males were used in all the experiments.

Islet Isolation and Treatment
Islets were isolated from mice pancreata through a combination of enzymatic and mechanical dissociation followed by purification on Histopaque 1077-1 Hybrid-Max (Sigma-Aldrich, St. Louis, MO, USA) gradients. Islets were incubated overnight in Roswell Park Memorial Institute 1640 medium (RPMI; Corning Incorporated, Corning, NY, USA) supplemented with 5 mM glucose. After this incubation period, islets were manually counted and sorted by size under a dissection microscope. Static insulin secretion experiments were performed using five handpicked, similarly sized islets obtained from the isolation. Selected islets were incubated overnight in 24-well plates with RPMI 1640 medium containing 10% FBS and 5 mM glucose. An insert was placed in each well to ensure that the islets remained localized to a central area of the well during stimulation. Before stimulation, islets were allowed to equilibrate for 1 hour at 37°C in basal condition (3mM glucose). After equilibration, islets were stimulated for 1 hour with either low or high glucose (3 mM and 16.7 mM, respectively), and 100 µM Oxo-M or 10 µM forskolin in the presence of 16.7 mM glucose. After stimulation, the supernatant was carefully extracted from the outer edges of the insert to ensure the islets would not be collected along and contaminate with the supernatant. The harvested supernatant was stored at -80°C for later analysis of insulin content by ELISA. Values obtained from the ELISA were normalized to the total amount of insulin measured in acid ethanol extracts.

Insulin ELISA
Insulin was measured with a sandwich ELISA kit (Mercodia, Uppsala, Sweden) as previously described (24).

MIN6 Culture
Two clones of MIN6 cells with passage numbers between 15-30 were used for these experiments: Gnb5 +/+ and Gnb5 -/- cells were created using the CRISPR-Cas9 system as previously described (24). The cells were maintained in culture at 37°C, 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies Corporation, Grand Island, NY, USA) containing 10% fetal bovine serum (VWR International, West Chester, PA, USA), 25mM glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µM tissue culture-grade beta-mercaptoethanol, 10 mM HEPES, and 10 mM sodium pyruvate.

MIN6 Cell Stimulation
For latrunculin B experiments, cells were plated in DMEM on 12-well plates at an approximate seeding density of 6x10^5 cells/well. After 24 hours, they were pre-incubated for 2 hours at 37°C in 3mM glucose in modified Krebs...
Ringer Buffer (KRB: 115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.19 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, 10 mM HEPES, pH 7.2). Cells are then stimulated with KRB containing either 16.7 mM glucose alone or together with 100 μM Oxo-M or 10 μM latrunculin B, for 30 minutes. Supernatant was collected and used for insulin ELISA.

For other stimulants, conditions were almost identical, but cells were plated on 24-well plates at an approximate seeding density of 5x10$^5$ cells/well. Stimulation times and reagent concentrations were dictated by the requirements of specific experiments.

**Studies of cell density effects**

MIN6 clones were plated in DMEM on 12-well plates at seedings densities of 3-, 6-, 9-, and 12x10$^5$ cells/well. After 24 hours, the cultures were evaluated for cell aggregate formation by phase-contrast microscopy using an inverted Nikon microscope with a 10x objective. Prior to stimulation, cells were pre-incubated for 2 hours at 37°C in 3mM glucose in modified KRB. Low glucose KRB was aspirated and replaced with either KRB containing 16.7 mM glucose alone or combined with 100 μM Oxo-M. After 30 minutes, supernatant was collected for insulin ELISA and the cells were harvested for Western blotting.

**Western blotting**

After supernatant was collected for insulin release, MIN6 cells were collected and subjected to SDS-PAGE and immunoblotting. Typically, we loaded 20 μg of total protein. After the transfer of proteins to nitrocellulose and incubation with primary and secondary antibodies, membranes were visualized using the Odyssey (Li-Cor Biosciences, Lincoln, NE, USA) infrared fluorescence system. For quantitative analysis, the signal in the band of interest (i.e. PKC substrates) was normalized to that for actin in the same lane.

**Mass Spectrometry (LC-MS/MS) of p28 band**

MIN6 cells were grown in 6-well plates to ~50% confluence, rinsed with HBSS (Thermo Fisher Scientific, Waltham, MA, USA) and incubated in serum-free DMEM for 1 h. They were then stimulated with 100 μM Oxo-M for 5 min; the medium was aspirated, and the cells were lysed in 0.5 ml of 1x SDS sample buffer. Samples were sonicated to destroy chromosomal DNA and used for gel electrophoresis. Proteins were resolved on either 10% Tris-glycine SDS gels/Tris-glycine running buffer or 10% bis-Tris SDS gels/MES running buffer (Thermo Fisher Scientific, Waltham, MA, USA).

In the initial experiment to locate the PKC substrates, the same samples were run in duplicates on the same gel, which was then cut in half. One half was stained with Coomassie Blue G250 to visualize proteins. The other was used for a western blotting with the rabbit polyclonal anti-phospho-Ser PKC substrate antibody. Both the Coomassie-stained gel and western blot were scanned using Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE, USA); 25 and 37 kDa protein standards were aligned, and the position of p28 PKC substrate band was determined relative to other Coomassie-stained proteins.

For the preparative gel, ~40 μg of total protein was loaded per lane into 9 lanes. After electrophoresis the gel was stained with Coomassie Blue G250, destained and left overnight in 15% EtOH/3% AcOH. Approximately 1 mm slices corresponding to the location of p28 band were excised from the gel. In addition, the bands right above and right below the p28 band were also cut out.

The gel slices were destained, reduced, alkylated, and digested with trypsin (53). The resulting peptides were extracted, reconstituted in 2% formic acid, and subjected to liquid chromatography and tandem mass spectrometry (LC-MS/MS) analysis (54).

LC MS/MS was performed with a Thermo Scientific LTQ Orbitrap Fusion Lumos Tribrid mass spectrometer equipped with an Ultimate Nano-LC system and a C-18 column (Acclaim PepMap, 75 μm × 15 cm, 2 μm, 100 Å). Five μL of the tryptic peptide solution were injected and eluted from the column using an acetonitrile, 0.1% formic acid gradient at a flow rate of 0.3 μL/min. The eluates were introduced into the source of the mass spectrometer on line. The microelectrospray ion source was operated at 2.3 kV. The digest was analyzed using the data-dependent multitask capability of the instrument.
acquiring full scan mass spectra from 300 to 1,700 Da at a resolution of 120,000. These mass spectra were followed by collision-induced dissociation experiments on the fifteen most abundant ions in the mass spectra. These collision-induced dissociation spectra were performed with collision energy of 28%. The products were analyzed in the Orbitrap mass spectrometer. Protein identification utilized Proteome Discoverer 1.4 (Thermo Fisher Scientific), the Mascot search engine (Matrix Science, 2.5), and the mouse Uni-Prot/Swiss-Protein database version (SwissProt 2016_07, 16,813 total mouse sequences). Database searches were restricted to ≤ 3 missed tryptic cleavage sites, precursor ion mass tolerance at 10 ppm, fragment ion mass tolerance at 0.02 Da, and a false discovery rate at 1%. Fixed modification was S-carbamidomethyl Cys, and variable modifications included Met oxidation, Asn and Gln deamidation, Ser and Thr phosphorylation with neutral loss, and Tyr phosphorylation.

**Statistics**

Data are presented as mean ± SD for the indicated number of experiments. Statistical significance was evaluated using single factor ANOVA. Data was considered significant at a value of P<0.05.

**Data Availability Statement:** All data presented and discussed are contained within the manuscript.
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† These authors contributed equally to this work.

Footnotes

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1The abbreviations used are: AVP, arginine vasopressin; GAP, GTPase-activating protein; GGL, G gamma-like; GLP-1, glucagon-like peptide; GPCRs, G protein-coupled receptors; GSIS, glucose-stimulated insulin secretion; M3R, muscarinic cholinergic receptor type 3; RGS, regulator of G protein signaling; Oxo-M, Oxotremorine-M.
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Gβ5-R7 facilitates glucose-stimulated insulin secretion

**Figure 1.** Gnb5 knockout inhibits stimulated insulin secretion in MIN6 cells. Gnb5 +/+ (grey bars) or Gnb5 -/- (open bars) MIN6 cells were stimulated with 10 μM latrunculin B (Lat), 100 μM Oxo-M or both, in the presence of 3 mM (3G, A) or 16.7 mM glucose (16G, B). The supernatants were collected for insulin ELISA analysis (Y axis). The data points show raw data from five independent cell culture experiments; each black dot is the average of the ELISA readings from triplicate wells. Bars show mean value with standard deviations. To avoid clutter in this figure, statistical analysis of the high glucose response is presented in Fig 2.
**Figure 2. Gβ5-R7 promotes GSIS in MIN6 cells and pancreatic islets.** MIN6 cells or pancreatic islets isolated from Gnb5 +/+ and Gnb5 -/- mice were incubated with 3 mM glucose before stimulation with 16.7 mM glucose. Supernatant was subjected to insulin ELISA. **A.** Data on the Gnb5 +/+ and Gnb5 -/- MIN6 cells from Fig. 1 are analyzed with single-factor ANOVA with n=5 (independent cell culture). **B.** Islets were prepared and treated as described in Experimental Procedures. Secretion of insulin was measured by ELISA using islets from three independent preparations. Bar graphs show mean ± SD. *P<0.05; **P<0.01; ***P<0.001; ns, not significant. Grey bars show Gnb5 +/+; white, Gnb5 -/-.
Figure 3. Gb5-R7 promotes insulin release evoked by several secretagogues in MIN6 cells and primary islets. A. Gnb5+/+ and Gnb5 -/- MIN6 cells were stimulated with 100 μM Oxo-M in the presence of 16.7 mM glucose or 50 mM KCl. B. Gnb5 +/- and Gnb5 -/- MIN6 cells were stimulated by 100 μM Oxo-M, 100 μM ADP, 0.1 μM arginine vasopressin (AVP), 0.1 μM GLP-1, or 10 μM forskolin all in the presence of 16.7 mM glucose. C. Islets isolated from Gnb5 +/- and Gnb5 -/- mice were stimulated with 100 μM Oxo-M in the presence of 16.7 mM glucose or 50 mM KCl. D. Islets were treated with 100 μM Oxo-M or 10 μM forskolin in the presence of 16.7 mM glucose. Values are reported as μg/L. Shown are raw ELISA readings, mean ± SD for at least five independent experiments. In C and D, n=3. Statistical analysis reported as the difference between 16G and each stimulant, within genotypes. *P<0.05; **P<0.01 ***P<0.001.
Figure 4. Gnb5 knockout reduces the impact of cell density on insulin secretion. A. Representative phase contrast images of cell culture at low and high density (3 x 10⁵ vs 9 x 10⁵ cells per well), at 100x magnification. Shown are Gnb5−/− cells, which are visually indistinguishable from controls. B. Representative immunoblot showing E-Cadherin expression in cultures of different densities and genotypes. Shown are samples at 6, 9 and 12 x 10⁵ cells per well. Equal amounts of cells were loaded, actin was used as a loading control. C. Gnb5+/+ (gray) or Gnb5 −/− (open) cells were plated at the 4 indicated densities in 12-well plates, stimulated with 16.7 mM glucose with or without 100 μM Oxo-M and secreted insulin was measured. ***P<0.001 D. Per well insulin secretion was normalized by the cell density to determine levels of individual cell secretion. Data points show average of six independent experiments. Error bars show SD.
Figure 5. Gnb5 knockout abrogates insulinotropic activity of collagen III and P7, agonist peptides of GPR56. Gnb5 +/+ and Gnb5 -/- cells were stimulated by 0.5 μM collagen III or 50 μM P7 in the presence of 16.7 mM glucose. Shown are raw ELISA readings from two independent cell culture experiments, mean ± SD. Statistical analysis reported as the difference between 16G and each stimulant, within genotypes. *P<0.05; **P<0.01 ***P<0.001.
Figure 6. PKC phosphorylates 14-3-3ε in MIN6 cells in a Gnb5-dependent manner. A. MIN6 cells were treated without or with 100 mM Oxo-M, and cell lysates were then analyzed by western blotting with anti-phospho-PKC substrate and b-tubulin antibodies. Note strong PKC-mediated phosphorylation of the protein with an apparent molecular mass 28 kDa (p28). This phosphorylation is increased ~2-fold in Gnb5-/- cells. Sizes of protein standards (in kDa) are indicated on the right. B. MIN6 lysates were subjected to electrophoresis using two different buffer systems –Tris-glycine and bis-Tris/MES running buffer. Migration of p28 in these systems is different relative to protein markers. C. MIN6 lysates were subjected to electrophoresis using two different buffer systems, and the gels were stained with Coomassie Blue G250. Areas of the gels corresponding to p28 (red rectangles) were cut out and used for the mass spectrometry analysis. In addition, areas right above and below (green and blue rectangles) were also analyzed by mass spectrometry. D. Confirmation of 14-3-3ε as p28 PKC substrate. MIN6 lysates were subjected to electrophoresis using bis-Tris/MES system. After the transfer the membrane was cut into three pieces and then analyzed by western blotting with either anti-phospho-PKC substrate or 14-3-3ε, or a mixture of two antibodies. Rabbit anti-phospho-PKC substrate was visualized in 680 nm channel (green); mouse anti-14-3-3ε antibody was visualized in 800 nm channel (red). E. The sequence of a 14-3-3ε phosphopeptide identified by the mass spectrometry analysis.
The regulatory G protein signaling complex, Gβ5-R7, promotes glucose- and extracellular signal stimulated insulin secretion
Qiang Wang, Taylor A. N. Henry, Alexey N. Pronin, Geeng-Fu Jang, Camila Lubaczeuski, John W. Crabb, Ernesto Bernal-Mizrachi and Vladlen Z Slepak

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