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β-cell tone is defined by proglucagon peptides through cyclic AMP signaling

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Abstract

Paracrine interactions between pancreatic islet cells have been proposed as a mechanism to regulate hormone secretion and glucose homeostasis. Here, we demonstrate the importance of proglucagon-derived peptides (PGDPs) for α- to β-cell communication and control of insulin secretion. Signaling through this system occurs through both the glucagon-like peptide receptor (Glp1r) and glucagon receptor (Gcgr). Loss of PGDPs, or blockade of their receptors, decreases insulin secretion in response to both metabolic and non-metabolic stimulation of mouse and human islets. This effect is due to reduced β-cell cAMP and affects the quantity but not dynamics of insulin release, indicating that PGDPs dictate the magnitude of insulin output in an isolated islet. In healthy mice, additional factors that stimulate cAMP can compensate for loss of PGDP signaling; however, input from α-cells is essential to maintain glucose tolerance during the metabolic stress induced by high-fat feeding. These findings demonstrate an essential role for α-cell regulation of β-cells, raising the possibility that abnormal paracrine signaling contributes to impaired insulin secretion in diabetes. Moreover, these findings support reconsideration of the role for α-cells in postprandial glucose control.
Introduction

Dysfunction of the pancreatic islet is central to the pathogenesis of diabetes, and insufficient insulin release from islet β-cells is the essential component for disease development (1). There is also evidence to support excess glucagon release from islet α-cells in persons with type 2 diabetes (T2DM) as a factor that contributes to hyperglycemia (2). Physiologic regulation of islet hormone secretion is generally attributed to plasma nutrients, particularly glucose and amino acids (AAs), as well as circulating insulinotropic hormones, termed incretins (3, 4). However, over the last several decades, communication between islet cell types has emerged as critical in the control of pancreatic endocrine function. For example, β-cells secrete several products that inhibit glucagon release from α-cells (5), and islet δ-cells serve a multifaceted role in paracrine inhibition of α- and β-cell secretion (6, 7). However, a key interaction that has not been explored to great depth is α- to β-cell communication, although several older, and recent, studies support the importance of this axis for β-cell function (8-13). β-cells express the glucagon receptor (Gcgr), a family-B G-protein coupled receptor (GPCR) that has significant homology and functional overlap with the receptors for the incretins, glucose-dependent insulinoactive polypeptide (GIP) and glucagon-like peptide 1 (GLP-1) (14). Moreover, it is well established that pharmacologic administration of glucagon stimulates insulin secretion (15-17), and recent work supports glucagon as a key species-specific determinant of glycemic set point (12). Despite this body of experimental data, the importance of paracrine stimulation of β-cell secretion by α-cells remains unclear, and the mechanism by which α-cells communicate with β-cells has not been conclusively established. Several recent papers suggest that α-cell production of GLP-1 mediates this signal (18, 19); yet previous observations that glucagon stimulates insulin secretion through both the β-cell Gcgr and GLP-1 receptor (Glp1r) (13, 15) adds a further layer of complexity to the process. Finally, it is unknown what physiological conditions would evoke α- to β-cell communication to stimulate insulin secretion. These gaps in knowledge, and the prominent role of circulating glucagon to promote hepatic glycogenolysis
and gluconeogenesis and raise blood glucose (20), have obscured paracrine actions of PGDPs on β-cells. Here, we demonstrate that: 1) α- to β-cell communication is necessary for a normal quantitative insulin response to both nutrient and non-nutrient β-cell depolarization; 2) PGDPs control the tone of insulin secretion by establishing β-cell levels of cyclic AMP (cAMP); 3) a subset of amino acids utilize PGDPs from α-cells to stimulate insulin secretion; and 4) PGDP input is necessary for the β-cell adaptations that maintain glucose tolerance in response to high-fat feeding.
Results

We sought to test the importance of α- to β-cell communication by generating a mouse line with a β-cell-specific deletion of the Gcgr (Gcgrpβcell-/--; Supplemental Figure 1A) (21). Islets isolated from littermate controls and Gcgrpβcell-/--; mice perifused with graded doses of glucagon displayed identical insulin secretion profiles (Figure 1A), suggesting that the Gcgr is dispensable for glucagon-stimulated insulin secretion. Consistent with these findings, Gcgrpβcell-/--; mice had glycemic excursions and glucose clearance comparable to WT controls in response to oral and intraperitoneal (i.p.) glucose challenges (Supplemental Figure 1, B and C). Moreover, a highly-specific Gcgr agonist failed to stimulate insulin secretion in Gcgrpβcell-/--; islets (Supplemental Figure 2A), confirming a functional Gcgr knockout. Since glucagon-stimulated insulin secretion can be blocked with an antagonist of the Glp1r (15), we pursued this alternative pathway for glucagon signaling. The Glp1r antagonist exendin-9 (Ex9) reduced glucagon-stimulated insulin secretion by ~65% in WT islets, and ~80% in Gcgrpβcell-/--; islets (Figure 1A), indicating that the Glp1r is the principal mediator of glucagon-stimulated insulin secretion. To validate this conclusion, we performed the reciprocal experiment using a glucagon receptor antagonist (GRA) and Glp1rβcell-/--; islets (22). In this experiment β-cell deletion of the Glp1r reduced glucagon-stimulated insulin secretion by ~ 75%, whereas the GRA in WT islets did not affect this (Figure 1B). However, the combination of GRA exposure in Glp1rβcell-/--; islets attenuated glucagon-stimulated insulin secretion to about 85% of normal. Together, these results corroborate previous reports that glucagon can stimulate insulin secretion through both the Gcgr and the Glp1r (13, 15), but show that glucagon signaling through the β-cell Glp1r is more important.

Although we tested a broad range of glucagon concentrations to measure glucagon-stimulated insulin secretion, it is not clear which of these are reflective of the concentrations within the islet, where paracrine α- to β-cell communication would take place. To gain insight into the paracrine effects of glucagon, we perifused islets with the AAs arginine and glutamine, which are known to stimulate
glucagon secretion (23-25). Both AAs stimulated α-cells to secrete glucagon (Figure 1C) and GLP-1 (Figure 1C, insets), but failed to stimulate insulin secretion from β-cells at low glucose (Supplemental Figure 2, B and C), indicating that these concentrations of AAs do not have a direct, glucose-independent effect on β-cells. While GLP-1 release from perifused α-cells is much less than glucagon (Figure 1C) it is ∼300 times more potent as an insulin secretagogue (Supplemental Figure 2, D and E). Therefore, it is possible that both peptides act as local insulinotropins in the islet, as previously reported (18, 19). To test the contribution of endogenously produced PGDPs for β-cell function, we perifused islets with AAs at high glucose and interrupted α- to β-cell communication using complementary strategies. AA-stimulated insulin secretion was intact in islets from Gcgrβcell−/− and Glp1rβcell−/− mice (Supplemental Figure 3, A and B), whereas pharmacological antagonism of either the Gcgr or Glp1r reduced AA-stimulated insulin secretion (Supplemental Figure 3, C and D). Remarkably, simultaneously blockade of both Gcgr and Glp1r using genetic knockouts and pharmacological antagonists nearly abolished AA-stimulated insulin secretion (Figure 1, D and E). These findings indicate that PGDPs are essential for AA-stimulated insulin secretion, that both the Gcgr and Glp1r can mediate this effect, and that compensation occurs for single receptor gene deletions that is not seen with acute pharmacologic blockade.

To further investigate α-cell regulation of β-cell function, we measured insulin secretion by islets from a line of mice with genetic disruption of Gcg expression; these animals lack production of all PGDPs including glucagon and GLP-1 (Gcg−/−) (26). Consistent with findings using interruption of Gcgr and Glp1r signaling, loss of the ligands for these receptors also severely blunted AA-stimulated insulin secretion (Figure 2A; Supplemental Figure 3E). Remarkably, islets from Gcg−/− mice also had dramatically reduced glucose-stimulated insulin secretion (GSIS) (Figure 2A). One explanation for this effect may be the loss of the acute rise in glucagon and GLP-1 release observed in WT islets after
transitioning from low to high glucose (3-8 minutes in Figure 1C), before the marked suppression of α-cell output typically seen with hyperglycemia (8-30 minutes in Figure 1C).

To determine whether germ-line deletion of Gcg leads to a general impairment of β-cell function, we stimulated insulin secretion with insulinotropic GPCR ligands: glucagon, GLP-1, and GIP. All three peptides stimulated insulin secretion comparably in WT and Gcg−/− islets (Figure 2A), demonstrating that provision of ligands for these GPCRs restores insulin secretion, and suggesting that the insulin secretory defect in the Gcg−/− is specific to loss of PGDPs. To confirm this, we used adenovirus-mediated delivery of Cre recombinase ex vivo to cleave the floxed stop codon in the proglucagon promoter of Gcg−/− islets and allow normal Gcg transcription (26). The Cre-mediated restoration of Gcg transcription within the islet restored α-cell synthesis of glucagon and GLP-1 to WT levels (Supplemental Figure 3F), and completely rescued glucose and AA-stimulated insulin secretion (Figure 2B); control virus (β-gal) treated Gcg−/− islets continued to display impaired nutrient-stimulated insulin secretion compared to WT islets (Supplemental Figure 3G). To confirm that the marked reduction of glucose- and AA-stimulated insulin secretion is the result of impaired α- to β-cell communication, rather than dysfunctional β-cells per se, WT islets were treated with Ex9 and GRA to block both the Glp1r and Gcgr acutely. Dual receptor blockade led to significant attenuation of glucagon-stimulated insulin secretion (Figure 2C), AA-stimulated insulin (Figure 2D), and GSIS (Figure 2, C and D), but not GIP-stimulated insulin secretion (Figure 2C). The normal response to GIP in islets treated with Ex9/GRA highlight that sufficient cAMP signaling can overcome the reduction in nutrient-stimulated insulin secretion brought about by antagonizing the receptors for PGDPs. Together, these findings demonstrate that PGDPs are necessary for nutrient-stimulated insulin secretion in isolated islets.

The current working model of GSIS divides the stimulatory effect of glucose into triggering (the effect of glucose to cause β-cell membrane depolarization and increase intracellular calcium) and amplifying (the effect of glucose to augment insulin release at a given calcium concentration) phases.
To determine if proglucagon-mediated β-cell signaling differentially impacts triggering versus amplification pathways (27), WT and Gcg<sup>-/-</sup> islets were perifused with diazoxide (Dz) and KCl at low glucose to assess triggering, or at high glucose to assess metabolic amplification (Figure 3). The insulin profiles from Gcg<sup>-/-</sup> islets had similar dynamics to controls, but markedly reduced insulin responses under both low and high glucose conditions. While both the triggering and amplifying components of insulin secretion were present, suggesting that the fundamental processes governing these events remained intact, the amount of insulin secreted was severely attenuated in the absence of PGDPs. This result suggests that signaling through the Gcgr and/or Glp1r controls the gain in β-cells, establishing an intracellular tone that governs quantitative insulin release.

The cardinal mechanism by which stimulatory family-B GPCRs, such as the Gcgr and Glp1r, signal is through G<sub>s</sub> activation of adenylyl cyclase, and production of cAMP. This led us to hypothesize that the reduced insulin secretion following impaired α- to β-cell communication is due to inadequate cAMP production. Indeed, cAMP antagonism with Rp-8-Br-cAMPS-pAB has been reported to decrease GSIS in human islets (28) and defective cAMP signaling contributes to impaired insulin secretion in Glp1r<sup>-/-</sup> islets (29) or in response to lipotoxicity (30). Consistent with our hypothesis, cAMP levels were decreased dramatically in β-cells acutely treated with Ex9/GRA, as determined by using a cAMP biosensor (Figure 4A). We also found that Ex9/GRA treatment reduced Ca<sup>2+</sup> levels during low-glucose, high-glucose, and KCl conditions (Figure 4B), along with a subtle reduction in the Ca<sup>2+</sup> responses to high-glucose or KCl (determined by the reduction in incremental AUCs for each condition). However, the Ca<sup>2+</sup> responses in islets treated with Ex9/GRA retained a similar pattern in the response to glucose or KCl compared with control conditions. A comparison of WT and Gcg<sup>-/-</sup> islets reveal a similar decrease in the Ca<sup>2+</sup> response to glucose, but not KCl in the knockout line (Figure 4C). The Ca<sup>2+</sup> responses in both models are compatible with the results of the Dz/KCl experiment (Figure 3), where lack of proglucagon input does not change the dynamics of insulin secretion regulated by changes in Ca<sup>2+</sup>, but
instead decreases the quantity of insulin secretion under all conditions, consistent with diminished β-cell tone. Moreover, the impaired insulin secretion in response to 30 mM KCl (Figure 3), despite sustained Ca\(^{2+}\) in Gcg\(^{-/-}\) islets (Figure 4C), illustrates that cAMP impacts other signaling pathways in addition to the regulation of Ca\(^{2+}\) levels. This also indicates that pathways independent of proglucagon signaling exert a significant influence on Ca\(^{2+}\) levels. To further validate the role of cAMP, we treated WT and Gcg\(^{-/-}\) islets with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) to prevent the degradation of intracellular cAMP. While IBMX activated PKA signaling in WT islets, both basal and stimulated PKA levels were significantly attenuated in Gcg\(^{-/-}\) islets (Figure 4D). Perfusion of WT islets with IBMX increased insulin secretion in a dose-dependent manner but had a minimal effect on Gcg\(^{-/-}\) islets (Figure 4E). To demonstrate that decreased insulin secretion from Gcg\(^{-/-}\) islets is due to insufficient cAMP levels, rather than impaired cAMP signaling, we perfused islets with increasing concentrations of the adenylyl cyclase activator forskolin (FSK). In this paradigm, both WT and Gcg\(^{-/-}\) islets had identical insulinotropic responses to increasing concentrations of FSK (Figure 4F). Taken together with the findings from experiments using GIP to stimulate insulin during the blockade of Gcgr and Glp1r (Figure 2C), this result suggests that alternative means of stimulating cAMP can compensate for the interruption of α- to β-cell signaling. To confirm this inference, we assessed exocytosis in individual patch-clamped β-cells from WT and Gcg\(^{-/-}\) islets in the presence or absence of cAMP. Notably, in the absence of cAMP, both WT and Gcg\(^{-/-}\) β-cells had similar, markedly reduced exocytosis (Figure 4G). However, the addition of cAMP to the patch pipette increased exocytosis to comparable levels in both sets of β-cells (Figure 4G). These results establish a mechanism whereby deficient cAMP signaling accounts for the severe defect in nutrient-stimulated insulin secretion observed in the absence of PGDPs. In total these experiments indicate that the attenuation of insulin secretion arising from impaired α- to β-cell communication can be ameliorated by mechanisms that augment cAMP production.
Human islet architecture has notable differences from rodent islets in ways that support greater \( \alpha/\beta \)-cell paracrine signaling \((31)\). First, \( \alpha \)-cells constitute a higher percentage of human islet cells, almost equal to \( \beta \)-cells, while the \( \alpha:\beta \) cell ratio in mice is closer to 1 to 4. Second, \( \alpha \)-cells are interspersed throughout the human islet \((31)\), with a large percentage of \( \alpha \)-cells juxtaposed to a neighboring \( \beta \)-cell; in mice \( \alpha \)-cells are situated on the periphery of the islet with less contact to \( \beta \)-cells. It is notable that human islets have higher insulin stimulated responses, particularly nutrients, compared to rodent islets \((3)\), and human islets transplanted into mice establish a lower ambient glycemia than rodent islets \((12)\). We tested the importance of \( \alpha \)- to \( \beta \)-cell communication in human islets using the pharmacological antagonists that were effective in mice and found that Ex9/GRA significantly inhibited AA- and glucose-stimulated insulin secretion \((\text{Figure 5A})\); these treatments also caused an acute rise in glucagon release \((\text{Supplemental Figure 4A})\). Notably, the combination of inhibitors is required to block glucagon-stimulated insulin secretion in human islets, as individual receptor blockade had limited effect \((\text{Supplemental Figure 4, B and C})\). Similar to murine islets, Ex9/GRA blunted both the triggering and metabolic amplification components of insulin release \((\text{Figure 5B})\). Also consistent with the results from mouse islets, IBMX could not potentiate insulin secretion normally in the presence of Ex9/GRA \((\text{Figure 5C})\), suggesting a defect in cAMP generation similar to what was observed in mice. And while GRA/Ex9 completely blocked glucagon-stimulated insulin secretion, the insulin response to GIP remained intact \((\text{Figure 5D})\), substantiating our mouse experiments which show that alternative activation of \( G_s/\text{adenylyl cyclase/cAMP} \) maintains insulin secretion.

Remarkably, Ex9/GRA treatment lowered insulin secretion in the early phases of our perifusions performed at basal glucose levels \((2.7 \text{mM})\), in the majority of human donors \((\text{Figure 5, A and C})\). To investigate this further, we added Ex9/GRA during extended perifusion of 2.7mM glucose and observed a dramatic decrease of insulin secretion \((\text{Figure 5E})\); insulin secretion remained lower as glucose concentrations were raised \((\text{Figure 5F})\). Moreover, glucagon, incretins, and AAs all stimulated insulin
secretion at low glucose concentration in human islets (Supplemental Data 4, F and G), a finding not seen in mouse islets (Supplemental Figure 2, B and C). Thus, in humans, α-cells have the potential to regulate β-cell tone across a wider range of glucose concentrations, including those mimicking fasting. In humans, fasting insulin secretion is proportional to systemic insulin sensitivity (32), an association that has not been explained. Our findings raise the possibility that α-cells link insulin needs with insulin provision.

To determine the effect of β-cell PGDP receptors in vivo, a setting where additional Gs GPCR ligands from outside the islet can contribute to the levels of cAMP, we generated mice with conditional deletion of Gcgr and Glp1r from the β-cell (Gcgr;Glp1rβcell−/−). Lean, chow-fed Gcgr;Glp1rβcell−/− mice had similar glucose tolerance to control mice in response to both i.p. and oral glucose loads (Figure 6, A and B), indicating that the β-cell receptors for PGDPs are dispensable in young, healthy mice. However, when Gcgr;Glp1rβcell−/− mice were chronically exposed to a high-fat diet and developed obesity, a different profile emerged. Metabolically stressed Gcgr;Glp1rβcell−/− mice were profoundly glucose intolerant in response to i.p. glucose compared with controls (Figure 6C). Moreover, fasting glycemia and the glucose excursion following an oral glucose challenge were also significantly higher in obese Gcgr;Glp1rβcell−/− mice compared to controls (Figure 6D), which had comparable body weights and levels of insulin sensitivity (Supplemental Figure 5). The somewhat muted difference during an oral glucose tolerance test compared with i.p. glucose led us to reason that a gut-derived GPCR ligand such as GIP could compensate for lack of PGDP signaling to preserve β-cell function.

We hypothesized that GIP sensitivity is enhanced in the islet of Gcgr;Glp1rβcell−/− mice and compensates for the lack of PGDP input, because Glp1r deletion has previously been shown to confer increased GIP sensitivity (33). To that end, we observed that Gcgr;Glp1rβcell−/− mice had a greater decrease of glycemia than controls when given exogenous GIP (Figure 7, A and B), and that perifused islets from Gcgr;Glp1rβcell−/− mice secreted more insulin in response to GIP (Figure 7C). Notably,
perifused islets from *Gcgr;Glp1rβcell−/−* mice phenocopied the impaired GSIS and amino acid-stimulated insulin secretion observed from *Gcg−/−* islets or islets treated with pharmacologic inhibitors. The compensatory increase in GIP sensitivity in *Gcgr;Glp1rβcell−/−* mice may be the factor preserving normal glucose tolerance in young, healthy mice. In this context it is important to consider that chronic hyperglycemia in T2D renders humans unresponsive to GIP, even at pharmacological levels (34), while also decreasing the expression of *GLP1R* and the insulin response to physiological levels of GLP-1 (35, 36), suggesting impaired β-cell signaling.
Discussion

The actions of GPCR ligands in the control of insulin secretion has been known for decades. The hallmark example of this is the incretin effect, attributed to GIP and GLP-1 and accounting for up to 70% of postprandial insulin secretion (37). Furthermore, it is commonly accepted that the incretin effect is significantly diminished in T2D (36), linking decreased GPCR signaling with insufficient insulin secretion and hyperglycemia. Over the past 2 decades new therapeutic agents that target incretin receptors on β-cells have been one of the major advances in diabetes care providing clinical evidence that activation of GPCRs on β-cells can improve insulin secretion and reduce glycemia. However, recent findings, including those presented here, challenge the conventional model of incretin action. This model holds that GPCR regulation of insulin secretion begins with nutrient-stimulated release of GIP and GLP-1 from enteroendocrine cells in the intestine, and emphasizes hormonal action of these gut-derived ligands on prandial insulin release (4). Recent evidence from preclinical models indicates that Glp1r activation is critical even in the absence of nutrient-stimulated GLP-1 (22) and that pancreatic rather than intestinal proglucagon re-expression Gcg−/− has the more important impact on glycemic regulation (26). These findings are consistent with local, paracrine actions of PGDPs, which include glucagon (Figure 1, A and B) (13, 15), as well as GLP-1 (Figure 1C), and potentially other PGDPs such as oxyntomodulin (38, 39). The findings presented herein define a new and expanded role for β-cell GPCR and identify PGDP as fundamental to regulated insulin secretion.

The major findings of this paper are that PGDPs are necessary for establishing the level of cAMP in β-cells, which determines the normal quantitative insulin response to both metabolic and non-metabolic stimuli. These results add to and extend the previous findings that demonstrate the importance of α-to β-cell communication (8, 40-42). These were the first reports that separating β-cells from the paracrine input of α-cells decreases insulin secretion and lowers cAMP levels. Subsequent reports measuring insulin secretion from single and paired cells by reverse haemolytic plaque assays showed
that α-β paired cells produced significantly more insulin in response to glucose that single β-cells alone (9). Finally, recent evidence has supported these ideas using isolated islets (39) or a perfused pancreas (13) in mouse models.

The results reported here have several important implications that extend this area of research. First, the insulotropic action of some AAs is indirect, and their stimulation of β-cells is mediated primarily through α-cell ligands for the Gcgr and Glp1r. This result suggests a distinct role for α-cells as islet AA sensors, and is consistent with recent observations of AA effects on α-cell growth and function (24, 25). In this model the α-cell is in central position to dictate the glucagon:insulin ratio in the portal circulation before and after meals, with significant implications for glucose homeostasis. Under fasting conditions, endogenously derived AAs arriving at the islet would result in a high glucagon:insulin ratio due to the reduced insulotropic action of PGDPs at low glycemia. This ratio would facilitate gluconeogenesis and endogenous glucose production. However, under postprandial conditions, AAs would stimulate both insulin and glucagon, significantly altering the glucagon:insulin ratio to favor glucose disposal.

Second, in the absence of PGDPs or Gcgr/Glp1r signaling, GSIS can be rescued by GIP or forskolin. These findings emphasize the importance of Gs/adenylyl cyclase/cAMP to permit the well-established effects of glucose to fully trigger, and amplify, insulin secretion (27). Importantly, our data extend the role of cAMP beyond potentiation of glucose-stimulated insulin secretion, which had been the prevailing view cAMP action in the insulin response (43). Indeed, our experiments show that the effects of cAMP are divorced from the Ca²⁺ signaling, revealing the potential for two distinct signaling pathways that govern insulin secretion. The first being metabolism of glucose that can both trigger a β-cell through depolarizing events that converge upon Ca²⁺ signaling (27) as well as dictate the level of insulin secretion through metabolic amplification (44). Our data describes a second, parallel pathway by which the level of cAMP dictates the magnitude of insulin secretion independent of the triggering and
amplifying pathways. In isolated murine and human islets, cAMP levels are dependent on PGDP input from α-cells, and this determines the amount of insulin released to a broad range of stimuli. Our data generally agrees with previous reports that show impaired GSIS in isolated islets devoid of α-cells (39) or in perfused pancreata with diminished PGDP signaling (13). Importantly, we demonstrate that the impaired β-cell function extends beyond GSIS. Indeed, based on the patterns of insulin released from perifused islets the general mechanisms of GSIS appear to be retained (Figure 3), but β-cell tone is diminished, and insulin profiles are diminished to a small fraction of those obtained when PGDP are present. Furthermore, the reduced β-cell tone mutes any stimuli that does not directly increase cAMP levels, including both metabolic (glucose, AAs) and non-metabolic (KCl, direct depolarization). Overall, these findings demonstrate a novel and expanded role for cAMP as a fundamental and essential mediator of β-cell function.

The effect of PGDP to drive the appropriate amount of insulin secretion translates to whole body physiology since mice with deletion of Gcgr and Glp1r have glucose intolerance in response to diet-induced obesity. This is a critical point in that the fundamental problem with diabetic β-cells is insufficient compensation for insulin resistance (45, 46); even very insulin resistant subjects can maintain normal glucose tolerance if insulin secretion is amplified to meet demands. In mice with β-cells that cannot respond to PGDP, insulin resistance induced by obesity causes a substantial disruption of glucose homeostasis. This observation indicates that PGDP are an essential component of the adaptation to metabolic stress and raises the possibility that abnormal α- to β-cell communication contributes to variation in glucose tolerance, and the development of diabetes.

In summary, we provide compelling evidence that PGDPs, acting through cAMP, determine β-cell tone, which directly influences the quantity of insulin secreted in response to glucose and AAs. Importantly, we demonstrate that in isolated human and murine islets, α-cell ligands for the Gcgr and
Glp1r are necessary for appropriate levels of cAMP in β-cells. In the absence of normal α- to β-cell signaling, metabolic adaptation to high-fat feeding and obesity is impaired causing glucose intolerance. Based on these results current models of the regulation of insulin secretion must be extended to include the essential contribution of α-cells and cAMP.
**Methods:**

**Reagents**

Glucagon and GLP-1 were purchased from Sigma and stocks prepared in 0.3% acetic acid. Mouse and human GIP were purchased from Chi Scientific and stocks prepared in PBS. All amino acids were purchased from Sigma and fresh stocks prepared in assay buffer for each experiment. Isobutylnmethylxanthine [IBMX] and forskolin [FSK] were purchased from Sigma and stocks prepared in DMSO. Exendin (9-39) [Ex9] was synthesized by GenScript and stocks were prepared in PBS.

Glucagon receptor antibody [GRA] in PBS was kindly provided by Kyle Sloop from Eli Lilly and Company (Indianapolis, IN). The glucagon receptor agonist 44-0410 was kindly provided by Brian Finan from Novo Nordisk (Indianapolis, IN).

**Animals**

Experiments were performed in 8-24 week old mice of the C57Bl6/J background. Mice were housed under a 12hr light/dark cycle and provided free access to a normal chow diet. Mice harboring LoxP sites in the Gcgr allele (GcgrFlox) (21, 47) were crossed with MIPcreERT (MIP-Cre) mice to generate β-cell specific deletion of Gcgr (GcgrCre/-). Briefly, MIP-CreCre/-:GcgrFlox/Flox were bred with MIP-CreCre/-:GcgrFlox/Flox mice to produce MIP-CreCre/-:GcgrFlox/Flox mice that were evenly divided to receive either oil (control) or tamoxifen (GcgrCre/-). In parallel breeding cages, MIP-CreCre/+ mice were bred to produce MIP-CreCre/+ and MIP-CreCre/+ mice that were evenly divided to receive either oil or tamoxifen. Consequently, our initial in vivo characterization of the GcgrCre/- mice included multiple control lines (MIP-CreCre/-:GcgrFlox/Flox + oil; MIP-CreCre/-:GcgrFlox/Flox + tamoxifen, MIP-CreCre/+ + oil or tamoxifen, MIP-CreCre/+ + oil or tamoxifen). Tamoxifen treatment consisted of 50mg/kg tamoxifen dissolved in corn oil administered in 6-week-old mice by oral gavage, and mice were used at least 1 month after induction. All control groups produced similar experimental results. For isolated islet experiments, control islets were from age-matched MIP-CreCre/+ treated with tamoxifen. Glp1rCre/- mice were generated as described previously (22) and followed a similar breeding strategy. Control islets for Glp1rCre/- mice were from age-matched MIP-CreCre/+ treated with tamoxifen. GcgrCre/- mice have been previously described (26). To generate experimental mice, GcgCre/+ mice were bred to produce GcgCre/+ (controls) and GcgCre/- mice. Gcgr;Glp1rCre/- mice were produced by breeding MIP-CreCre/-:GcgrFlox/Flox;Glp1rCre/-:GcgFlox/Flox and MIP-CreCre/-:GcgrFlox/Flox;Glp1rCre/-:GcgFlox/Flox mice. Controls (MIP-CreCre/+ and Gcgr;Glp1rCre/- mice were given the same tamoxifen protocol described above. We choose to use MIP-CreCre/+ mice treated with tamoxifen as our primary control given our previous report that the Mip-Cre transgene produces a phenotype in the setting of high-fat diet + streptozotocin (49).

However, we have also recently reported that MIP-CreCre/+ mice have similar β-cell function and glucose tolerance compared to MIP-CreCre/+ mice in conditions absent streptozotocin when treated with either oil or tamoxifen(50), consistent with the findings present by Oropeza et al (49). Male mice were used for all physiology experiments, and both male and female mice were used for islet experiments. A separate analysis of male versus female islets indicate there were no effects of sex on any secretion parameters.

**Glucose- and Meal-tolerance Tests**

Oral and intraperitoneal glucose-tolerance tests (GTTs) or meal-tolerance tests (MTTs) were performed in mice after a 5hr fast. Glucose was administered at 1.5g/kg in PBS and liquid Ensure orally administered at 10ml/kg, and glucose was measured using a glucometer (Contour). D-Ala GIP was
administered i.p. at 4 nmol/kg. For fast-refeed experiments, mice were fasted for ~16hrs and blood was collected at baseline and then 30 and 60min after re-introduction of chow diet into the cage. EDTA-coated capillary tubes were used to collect blood. Mice were fed a high fat diet (HFD) containing 45 kcal% fat (Research Diets) ad libitum for 8-weeks prior to i.p. and oral GTTs.

**Islet Isolation**

Primary islets were isolated from mice according to previously published methods (51). Briefly, the pancreas was inflated through the pancreatic duct with 0.8mg/mL collagenase V in HBSS. The pancreas was then excised and digested for 12 min at 37°C. Digestion was quenched with cold RPMI (2mM L-glutamine, 11.1mM glucose, 0.25% BSA, 100U/ml penicillin, and 100µg/ml streptomycin). Islets were separated using a Histopaque gradient. Islets recovered overnight in RPMI containing 10% FBS prior to all experiments.

**Islet Perfusion**

After incubation, equal numbers of islets (75-100 islets) were hand-picked and placed into chambers containing 2.7mM glucose KRPH buffer (140mM NaCl, 4.7mM KCl, 1.5mM CaCl₂, 1mM NaH₂PO₄, 1mM MgSO₄, 2mM NaHCO₃, 5mM HEPES, and 1% FA-free BSA; pH=7.4) with 100 µL Bio-Gel® P-4 Media (Bio-Rad). Islets were equilibrated for 48 minutes, and then perifused in intervals based on the experimental conditions. All treatments were prepared in KRPH buffer. Insulin and glucagon content and secretion was assessed by AlphaLISA (Perkin Elmer) and assayed using the EnVision plate reader (Perkin Elmer). Total GLP-1 content and secretion was measured by ELISA (Meso Scale) and measured with a SECTOR 2400 imager (Meso Scale).

**Viral Reactivation of Proglucagon Products**

Recombinant Ad-CMV-Cre (ID# HM101) and Ad-CMV-βgal (ID# HD701) were generated using a new modular cloning platform, pMVP, that are described elsewhere (52). In brief, cDNA for Cre and βgal were PCR amplified to incorporate attB4r/attB3r sites, and subsequently recombined into pDONR221 P4r-P3r (Invitrogen) using BP Clonase II per the manufacturer’s protocol (Invitrogen) to form MultiSite Gateway Pro entry plasmids. These were then recombined with MultiSite Gateway Pro entry plasmids containing the CMV promoter and IRES2-eGFP followed by the SV40 polyadenylation signal into the adenovirus backbone pAd/PL-DEST (Invitrogen) via an overnight reaction mediated by LR Clonase II plus (Invitrogen). The reaction was then transformed into NEB 10-beta competent cells (New England Biolabs) and clones containing the final adenovirus vector were isolated and validated by diagnostic restriction digests. Recombinant adenoviral plasmids were subsequently linearized with PacI, propagated in HEK293 cells, and purified using CsCl₂ gradients. Purified adenovirus particles were titered by A₂₆₀ and determined to be E1A deficient using a qRT-PCR screen. Immediately after islets were isolated, they were placed in RPMI containing 1µL/ml virus for 24hrs. Islets were then allowed to recover for 48-72hrs in RPMI before being perifused.

**Western Blot Analysis**

Approximately 200-250 islets were incubated at 37°C in 2.7mM glucose KRPH buffer for six hours. Islets were then treated with either vehicle or 100µM IBMX dissolved in 2.7mM glucose KRPH buffer for 5 min. Islets were washed, lysed and frozen overnight at -20°C. On the following day, lysates were run in BCA assay (ThermoFisher). Membranes were blocked in 5% non-fat TBST (Tween 20) milk and
incubated with either Phospho-PKA substrate (Cell Signaling, #9624) or Hsp90 (Cell Signaling, #4877) antibody overnight at 4°C. Images were analyzed using Image Lab software (BioRad).

Cloning and Adenoviral Delivery of cAMP Biosensors into Islet β-cells

The cDNA for cAMP biosensor (Epac-S\textsuperscript{H189}, $K_d = 4 \, \mu M$) was cloned (53) by Gibson Assembly (New England Biolabs) into a modified pENTR-DS shuttle vector (Invitrogen) containing the rat insulin promoter (RIP) and rabbit β-globin intron as in a previous study (54). ClonaseII/Gateway (Invitrogen) was then used to prepare the full-length adenoviral construct in pAd/PL-DEST (Invitrogen), yielding β-cell specific cAMP biosensors (pAd-RIP1-Epac-S\textsuperscript{H189}-pA). Islets were infected immediately post-isolation with 1.5μl of high-titer adenovirus for 2hrs at 37°C, then moved to fresh media overnight.

Cytoplasmic Ca\textsuperscript{2+} and cAMP Imaging

Islets from wild-type or Gcg\textsuperscript{null} mice were imaged simultaneously; one group was pre-labeled with 1μg/mL DiR (Molecular Probes, Eugene, OR) for 10 minutes. DiR labeling had no effect on islet metabolic or Ca\textsuperscript{2+} oscillations (data not shown). For measurements of cytoplasmic Ca\textsuperscript{2+}, islets were either pre-incubated in 2.5μM FuraRed (2.5μM, 45 min; Molecular Probes F3020) or Calbryte590 (2.5μM, 60 min; AAT Bioquest 20700) in islet media at 37°C before they were placed in a glass-bottomed imaging chamber (Warner Instruments) mounted on a Nikon Ti-Eclipse inverted microscope equipped with a 10X/0.50NA SuperFluor objective (Nikon Instruments). The chamber was perfused with a standard external solution containing 135mM NaCl, 4.8mM KCl, 2.5mM CaCl\textsubscript{2}, 1.2mM MgCl\textsubscript{2}, 20mM HEPES (pH 7.35). The flow rate was maintained at 0.25mL/min using feedback control (Fluigent MCFS-EZ) and temperature was maintained at 33°C using solution and chamber heaters (Warner Instruments). Excitation was provided by a SOLA SEII 365 (Lumencor) set to 10% output. Single DiR images utilized a Chroma Cy7 cube (710/75x, T760lpxr, 810/90m). For FuraRed, excitation (430/20x and 500/20x, ET type, Chroma Technology Corporation) and emission (650/60m) filters (BrightLine type, Semrock) were used in combination with an FF444/521/608-Di01 dichroic (Semrock) and reported as an excitation ratio (R430/500). The same dichroic was used for Calbryte 590, but an ET572/32x filter was used for excitation and an ET632/60m filter was used for emission. The same dichroic mirror was used for cAMP biosensor FRET imaging, with CFP excitation provided by an ET430/24x filter and emission filters for CFP and Venus emission (ET470/24m and ET535/30m, Chroma) reported as an emission ratio (R470/535). Fluorescence emission was collected with a Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera every 6 seconds. A single region of interest was used to quantify the average response of each islet using Nikon Elements.

Capacitance Measurements

Islets were dispersed in calcium-free dissociation buffer and incubated overnight in 11mM glucose RPMI. Patch clamping was performed using the standard whole-cell technique with the sine + DC lock-in function of an EPC10 amplifier and Patchmaster software (HEKA Electronics). Experiments were performed at 32-35°C using an extracellular bath solution (118mM NaCl, 20mM TEA, 5.6mM KCl, 1.2mM MgCl\textsubscript{2}, 2.6mM CaCl\textsubscript{2}, 5mM glucose, 5mM Hepes, pH = 7.4) and pipette solution 125mM CsGlutamate, 10mM CsCl, 10mM NaCl, 1mM MgCl\textsubscript{2}, 0.05mM EGTA, 5mM Hepes, 3mM MgATP, pH -7.15) which contained either 0.1mM cAMP or no cAMP. Capacitance response to a train of 10 500-ms depolarizations (-70 to 0mV) were normalized to initial cell size and expressed as femtofarad per picofarad (fF/pF). Mouse β cells were identified by size and the inactivation of a voltage-gated Na\textsuperscript{+} current at around -90mV.
Statistical Analysis

All data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 7. A 2-tailed Student’s t-test, one-way, or two-way ANOVA were performed, depending on the experimental design, with a Bonferroni post-hoc analysis. p<0.05 was determined to identify statistically significant differences.

Study Approval

All mouse procedures were approved and performed in accordance with the Duke University Institutional Animal Care and Use Committee.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.
References:


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Figure 1. Proglucagon products stimulate insulin secretion through both the Glp1r and Gcgr. (A) Insulin secretion in response to increasing doses of glucagon in Control (Con; MIP-CreERT) or Gcgrβcell−/− islets ± 1µM Exendin 9-39 (Ex9). ( , ; n=9, 8, 3, 7) (B) Insulin secretion in response to increasing doses of glucagon from Con or Glp1rβcell−/− islets ± 10µg/ml GRA. ( , ; n=6, 6, 5, 5) (C) Glucagon and total GLP-1 secretion in response to 10mM glutamine and 1mM arginine. (n=3) (D) Insulin secretion in response to 10mM glutamine and 1mM arginine from Con islets or Glp1rβcell−/− treated with 1µM Ex9. (n=6) (E) Insulin secretion in response to 10mM glutamine and 1mM arginine from WT islets or Glp1rβcell−/− treated with 10µg/ml GRA. (n=5) *p<0.05 as indicated. (n=6) Data are shown as mean ± SEM. Data was analyzed with a two-way ANOVA for the iAUCs (A, B, D, E) or a 2-tailed Student’s t-test (C).
Figure 2. Proglucagon products are necessary for nutrient-stimulated insulin secretion. (A) Insulin secretion in response 10mM glucose, 10mM glutamine, 1mM arginine, 10nM glucagon, 3nM GIP, and 0.3nM GLP-1 from WT or Gcg−/− islets (n=7). (B) Insulin secretion in response 10mM glucose, 10mM glutamine, 1mM arginine, 10nM glucagon, 3nM GIP, and 0.3nM GLP-1 from WT + Ad-CMV-Cre (n=3) or Gcg−/− + Ad-CMV-Cre islets (n=5). (C) Insulin secretion in response to 10mM glucose, 10nM glucagon, and 3nM GIP from WT islets ± 1µM Ex9 and 10µg/ml GRA (n=6). (D) Insulin secretion in response to 10mM glucose, 10mM glutamine, and 1mM arginine from WT islets ± 1µM Ex9 and 10µg/ml GRA (n=6). *p<0.05 as indicated. Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of the iAUCs.
Figure 3. Lack of proglucagon peptide input reduces insulin secretion in response to both triggering and amplification signals. Insulin secretion in response to different concentrations of 2.7 mM glucose, 10 mM glucose, 400 µM diazoxide (Dz), or Dz with 30mM KCl, as indicated from WT (n=5) or Gcg<sup>−/−</sup> islets (n=6). Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of the iAUCs or a 2-tailed Student’s t-test (inset).
Figure 4. Impaired proglucagon input reduces cAMP signaling in β-cells. (A) Average cAMP levels from WT islets acutely exposed to either control (n=21) or Ex9/GRA (n=24) conditions. (B) Cytosolic Ca^{2+} levels in WT islets acutely exposed to either control (n=21) or Ex9/GRA (n=24) conditions in response to 10mM glucose or 30mM KCl. (C) Cytosolic Ca^{2+} levels in WT (n=30) and Ggc^−/− (n=21) islets in response to 10 mM glucose or 30 mM KCl. (D) Phosphorylation of PKA substrates and HSP90 protein levels in WT (n=7) or Ggc^−/− islets (n=7). (E) Insulin secretion in response to increasing doses of IBMX in WT (n=6) or Ggc^−/− islets (n=6) at 10mM glucose. (F) Insulin secretion in response to increasing doses of FSK in WT or Ggc^−/− islets (n=3) at 10mM glucose. (G) Cumulative capacitance from sequential depolarization in individual β cells from WT or Ggc^−/− islets ± cAMP (left); representative trace of depolarizations (right). (●, ○, ▲, △; n=38, 38, 34, 37) *p<0.05 as indicated. Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of the iAUCs.
Figure 5. Proglucagon products set the tone for insulin secretion in human islets. (A) Insulin secretion from human islets ± 1µM Ex9 and 10µg/ml GRA stimulated with 10mM glutamine or 1mM arginine (n=3). (B) Insulin secretion in response to different combinations of 2.7mM glucose, 10mM glucose, 1µM diazoxide (Dz), or Dz with 30mM KCl, as indicated from human islets ± 1µM Ex9 and 10µg/ml GRA (n=3). (C) Insulin secretion in response to increasing doses of IBMX in human islets ± 1µM Ex9 and 10µg/ml GRA (n=3). (D) Insulin secretion from human islets ± 1µM Ex9 and 10µg/ml GRA (n=3) stimulated with 10nM glucagon or 50nM GIP. (E) Insulin secretion from human islets ± 1µM Ex9 and 10µg/ml GRA (n=3) in response to low glucose. (F) Insulin secretion from human islets in response to increasing glucose concentrations ± 1µM Ex9 and 10µg/ml GRA (n=3). *p<0.05 as indicated. Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of the iAUCs (A, B, D, F) or a 2-tailed Student’s t-test (C).
Figure 6. Loss of PGDP input into β-cell combined with high-fat feeding leads to glucose intolerance. (A) i.p. glucose tolerance (1.5mg/kg) and iAUC of 12-16-week old chow fed control (Con; MIP-CreERT, n=10) and Gcgr:Glp1rβcell−/− (n=13) mice. (B) Oral glucose tolerance (1.5mg/kg) and iAUC of 12-16-week chow fed control (n=9) and Gcgr:Glp1rβcell−/− (n=14) mice. (C) i.p. glucose tolerance (1.5mg/kg) and iAUC of 20-24-week old HFD fed control (n=5) and Gcgr:Glp1rβcell−/− (n=10) mice. (D) Oral glucose tolerance (1.5mg/kg) and iAUC of 20-24-week old HFD fed control (n=5) and Gcgr:Glp1rβcell−/− (n=10) mice. *p<0.05 vs control as indicated. Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of glycemic curves or a 2-tailed Student’s t-test of the iAUCs.
Figure 7. Gcgr:Glp1rβcell−/− mice show an increased sensitivity to GIP in vivo and ex vivo. (A) i.p. glucose tolerance and iAUC from control (n=11) and Gcgr:Glp1rβcell−/− (n=13) mice on a chow-diet treated with PBS or D-Ala-GIP (4nmol/kg) 10 min before glucose (1.5mg/kg). (B) Glycemia in ambient fed control (n=9) and Gcgr:Glp1rβcell−/− (n=14) mice on chow diet after i.p. injection of PBS or D-Ala-GIP (4nmol/kg). (C) Insulin secretion in response 10mM glucose, 10mM glutamine, 1mM arginine, 10mM glucagon, 3nM GIP, and 0.3nM GLP-1 from control (n=7) or Gcgr:Glp1rβcell−/− islets (n=6). *p<0.05 as indicated. Data are shown as mean ± SEM. Data was analyzed by a two-way ANOVA of glycemic curves (A, B) and the iAUCs.