Intra-islet glucagon confers β-cell glucose competence for first-phase insulin secretion and favors GLP-1R stimulation by exogenous glucagon

We report that intra-islet glucagon secreted from α-cells signals through β-cell glucagon and GLP-1 receptors (GcgR and GLP-1R), thereby conferring to rat islets their competence to exhibit first-phase glucose-stimulated insulin secretion (GSIS). Thus, in islets not treated with exogenous glucagon or GLP-1, first-phase GSIS is abolished by a GcgR antagonist (LY2786890) or a GLP-1R antagonist (Ex[9–39]). Mechanistically, glucose competence in response to intra-islet glucagon is conditional on β-cell cAMP signaling because it is blocked by the cAMP antagonist prodrug Rp-8-Br-cAMPS-pAB. In its role as a paracrine hormone, intra-islet glucagon binds with high affinity to the GcgR, while also exerting a “spillover” effect to bind with low affinity to the GLP-1R. This produces a right shift of the concentration-response relationship for the potentiation of GSIS by exogenous glucagon. Thus, 0.3 nM glucagon fails to potentiate GSIS, as expected if similar concentrations of intra-islet glucagon already occupy the GcgR. However, 10 to 30 nM glucagon effectively engages the β-cell GLP-1R to potentiate GSIS, an action blocked by Ex[9–39] but not LY2786890. Finally, we report that the action of intra-islet glucagon to support insulin secretion requires a step-wise increase of glucose concentration to trigger first-phase GSIS. It is not measurable when GSIS is stimulated by a gradient of increasing glucose concentrations, as occurs during an oral glucose tolerance test in vivo. Collectively, such findings are understandable if defective intra-islet glucagon action contributes to the characteristic loss of first-phase GSIS in an intravenous glucose tolerance test that is diagnostic of type 2 diabetes in the clinical setting.

Pancreatic β-cells located in the islets of Langerhans serve as blood glucose sensors in that they secrete insulin in response to the rise of blood glucose concentration that occurs after a meal (1). This glucose-stimulated insulin secretion (GSIS) requires glucose uptake and oxidative glucose metabolism that generates key ionic (Ca2+) and metabolic coupling factors (ATP, glutamate, NADPH, and monoacylglycerol) that act intracellularly to promote exocytosis of insulin packaged within large dense core secretory granules (2). Stimulus-secretion coupling of this type is of high importance to the maintenance of systemic glucose homeostasis by virtue of the fact that circulating insulin promotes glucose uptake while also inhibiting glucose release in target tissues that express insulin receptors (3). Thus, defective GSIS leads to insulin insufficiency that predisposes to hyperglycemia with consequent transition to type 2 diabetes (T2D) (4).

In humans intravenously infused with glucose, it is possible to study the kinetics of GSIS in response to a step-wise increase of blood glucose concentration. This approach reveals two distinct kinetic components of GSIS that are defined as first and second phase insulin secretion. Remarkably, the loss of first-phase GSIS is an accurate predictor of T2D at early stages in the disease process (5–7). Why this is the case is not known, but an attractive hypothesis is that the “competence” of β-cells to release insulin in response to glucose is conditional on an intra-islet paracrine hormone signaling mechanism. In this scenario, glucagon released from islet α-cells acts at β-cell glucagon receptors (GcgR) and/or glucagon-like peptide-1 receptors (GLP-1R) to stimulate the production of cAMP that acts as a necessary cofactor to support GSIS (8–20), thereby maintaining “β-cell tone” (15). Thus, a diminished intra-islet paracrine hormone action of glucagon might lead to a loss of β-cell glucose competence manifest as a loss of first and/or second phase GSIS. Intriguingly, this defect might be corrected by β-cell cAMP-elevating agents such as dulaglutide or semaglutide that are commonly prescribed for treatment of T2D (21–24).

What has not been established to date is whether intra-islet glucagon acts through the GcgR and/or GLP-1R to exert a selective effect to upregulate first and/or second phase GSIS. A selective effect is possible based on our previous report that the cAMP antagonist prodrug Rp-8-Br-cAMPS-pAB abolished first-phase but not second-phase GSIS in response to glucose alone (25). Rp-8-Br-cAMPS-pAB is a highly membrane permeable para-acetoxybenzyl (pAB) ester prodrug that is bioactivated by cytosolic esterases to liberate unconjugated
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Rp-8-Br-cAMPS that competitively inhibits stimulatory effects of endogenous cAMP at the cAMP-binding domains of cAMP-dependent PKA (25) and cAMP-regulated guanine nucleotide exchange factors Epac1 and Epac2 (25). In this regard, PKA (26–31) and Epac2 (29, 31–39) are the principal targets of cAMP relevant to β-cell insulin secretion.

To determine if intra-islet glucagon exerts its secretagogue action primarily at the GcgR, we treated islets with the GcgR antagonist (GRA) monoclonal antibody LY2786890 (a.k.a., Ab-4) (40). Because the volume-restricted microenvironment of islets may allow glucagon to accumulate at concentrations high enough to stimulate the GLP-1R (41), we also tested the GLP-1R antagonist Exendin-[9–39] (42, 43). Complementary studies using FRET assays to detect cAMP, or immunoassays to detect glucagon, allowed formulation of a new receptor occupancy model of glucagon action that explains the previously reported paradoxical findings that the GLP-1R mediates actions of exogenous glucagon to stimulate insulin secretion (9, 44). Finally, we report that the action of intra-islet glucagon to support insulin secretion requires a step-wise increase of glucose concentration to trigger first-phase GSIS. It is not measurable when GSIS is stimulated by a gradient of increasing glucose concentrations, as occurs during an oral glucose tolerance test (OGTT) in vivo (45). Collectively, such findings are understandable if defective intra-islet glucagon action contributes to a characteristic loss of first-phase GSIS in the intravenous glucose tolerance test that is diagnostic of T2D in the clinical setting (5).

Results

Differential control of first and second phase GSIS by glucose and GLP-1

Intra-islet hormones may act as endogenous competence factors to enable GSIS in response to glucose alone. Furthermore, their presence in islets may alter the potency and efficacy of glucagon or GLP-1[7–36]amide (GLP-1) when each is administered as a synthetic peptide. To investigate these possibilities, we established methods that allow automated sampling for fast temporal resolution of first and second phase GSIS. Initially, these methods were validated in perfusion assays using GLP-1R agonists and antagonists, thus revealing novel features concerning GLP-1 insulin secretagogue action. Such studies provided a baseline for subsequent assays using GcgR agonists and antagonists so that the pharmacological properties of GLP-1 and glucagon could be compared.

Perfusion assays using batch preparations of islets obtained from Sprague–Dawley rats demonstrated the expected potentiation of first and second phase GSIS in response to administered GLP-1 (Fig. 1, A1 and A2). This action of GLP-1 was measured under conditions in which the glucose concentration was increased in a step-wise manner from 2.8 to 16.7 mM. Area-under-the-curve (AUC) analysis of Fig. 1, B1–B3 revealed that the threshold GLP-1 concentration for potentiation of first-phase GSIS was ca. 100 pM (Fig. 1B1). In contrast, the threshold for potentiation of second-phase GSIS was ca. 1 nM (Fig. 1B2). Thus, we obtained the novel finding that first-phase GSIS was responsive to a tenfold lower concentration of GLP-1 in comparison with second-phase GSIS. As explained in greater detail below, the high potency of administered GLP-1 is expected if levels of intra-islet GLP-1 are so low that there is little or no prior occupancy of the β-cell GLP-1R by endogenous GLP-1. Importantly, the specificity with which GLP-1 exerted its secretagogue action in this assay was established based on the ability of GLP-1R antagonist Ex [9–39] to block the agonist action of GLP-1 (Fig. 1, C1 and C2).

Next, we confirmed our previous report (25) that the cAMP antagonist produg Rp-8-Br-cAMPS-pAB (10 μM) blocked first-phase GSIS in response to glucose alone, while having little ability to reduce second-phase GSIS in response to glucose alone (Fig. 2, A1 and A2). We also found that GLP-1 (1 nM) failed to restore first-phase GSIS in rat islets treated with Rp-8-Br-cAMPS-pAB (Fig. 2, B1 and B2). Of interest, we obtained the novel finding that GLP-1 enabled cAMP-dependent insulin exocytosis to become operational during second-phase GSIS (Fig. 2B1). Thus, Rp-8-Br-cAMPS-pAB greatly reduced the amplitude of second-phase GSIS in the presence of GLP-1 (Fig. 2B1). Note that the residual second-phase GSIS measured during treatment with Rp-8-Br-cAMPS-pAB was of similar magnitude to that measured in the absence of GLP-1. This novel finding reveals that during second-phase GSIS, there is a summation of cAMP-dependent and cAMP-independent processes of insulin exocytosis, each under the control of GLP-1 and glucose, respectively. We also provide evidence that Epac2 and PKA participate in the control of GSIS from rat islets. This concept is consistent with our finding that the first and second phases of GSIS were potentiated by cyclic nucleotide analog prodrugs that are selective activators of Epac (8-pCPT-2′-O-Me-cAMP-AM) or PKA (6-Bnz-cAMP-AM) (Fig. 2, C1 and C2).

To test if Rp-8-Br-cAMPS-pAB blocks Epac and PKA activation, we used the rat INS-1 832/13 insulin-secreting β-cell line that expresses the endogenous GLP-1R (46). These cells were virally transduced with the FRET reporter H188 that contains the cyclic nucleotide-binding domain of Epac (47). GLP-1 raised the levels of cAMP (EC\textsubscript{50} 1.9 nM) (Fig. 2, D1 and D2), an effect blocked by Rp-8-Br-cAMPS-pAB (IC\textsubscript{50} 1.4 μM) (Fig. 2, E1 and E2). The cells were also transduced with the FRET reporter AKAR3 that is a substrate for PKA and that serves as an indirect readout for binding of cAMP to PKA regulatory subunits (48). GLP-1 stimulated PKA activity (EC\textsubscript{50} 88 pM) (Fig. 2, F1 and F2), an effect blocked by Rp-8-Br-cAMPS-pAB (IC\textsubscript{50} 2.8 μM). Thus, Epac and PKA activation by cAMP is blocked by Rp-8-Br-cAMPS-pAB.

Having established the suitability of Rp-8-Br-cAMPS-pAB as a tool for evaluation of cAMP signaling in β-cells, a more detailed analysis of its antagonist action was performed across a range of GLP-1 concentrations in assays of GSIS. Consistently, Rp-8-Br-cAMPS-pAB blocked the potentiation of first and second phase GSIS when GLP-1 was tested at concentrations of 5, 1, and 0.1 nM (Fig. 3, A–D). Interestingly, the antagonist action of Rp-8-Br-cAMPS-pAB was weaker versus 5 nM GLP-1 in comparison with 1 nM GLP-1 (cf., Fig. 3, A and B). This is expected because free Rp-8-Br-cAMPS competes with
endogenous cAMP for binding to Epac and PKA, thereby rendering this cAMP analog less effective when concentrations of cAMP rise to high levels in response to 5 nM GLP-1. Because bioactive GLP-1 circulates at low concentrations, it is of interest that Shigeto et al. reported an ability of extremely low concentrations of GLP-1 (0.1–10 pM) to stimulate mouse islet insulin secretion, an effect they explained by PLC and PKC activation (49–51). In contrast, we obtained the novel finding that concentrations of GLP-1 less than 100 pM failed to stimulate rat islet insulin secretion (Fig. 1, A1 and A2), while also failing to stimulate mouse islet insulin secretion using the methods of Shigeto et al. (Fig. S1). Using PLC and PKC inhibitors tested by Shigeto et al., we were also unable to confirm PLC- or PKC-mediated actions of GLP-1 to potentiate GSIS from rat islets (Fig. S2). PLC inhibitor U73122 alone potentiated GSIS, an effect not reproduced by the structurally related U73343 that is not a PLC inhibitor, (Fig. S2). Furthermore, GLP-1 retained its ability to potentiate GSIS during treatment with U73122 or U73343 or PKC inhibitors LY 333551 and Ro 31-8220 (Fig. S2). Still, prior studies demonstrate a novel cAMP signaling mechanism that is stimulated by Epac2 in mouse islets, and that engages PLCε to upregulate PKC activity, IP3 production, Ca2+ signaling, and insulin secretion (52–54). Because this nonconventional cAMP signaling is blocked by Rp-8-Br-cAMPS acting at Epac2, it might participate in the control of GSIS by GLP-1, a possibility that remains to be explored in future studies.

**Intra-islet glucagon reduces the potency of administered glucagon in assays of GSIS**

The above-summarized pharmacological properties of GLP-1 were compared with those of glucagon in additional assays of GSIS. Unexpectedly, low or intermediate concentrations of glucagon (0.3–3 nM) failed to potentiate GSIS (Fig. 4, A1 and A2). AUC analysis revealed that the threshold glucagon concentration for potentiation of first-phase, second-phase, and total GSIS was ca. 3 to 10 nM (Fig. 4, B1–B3). Still, higher concentrations of glucagon (10–300 nM) exerted a significant

**Figure 1. Concentration-response relationship for potentiation of GSIS by GLP-1 in rat islets.** A1 and A2, GLP-1 potentiated GSIS that was initiated by a step-wise increase of glucose concentration from 2.8 to 16.7 mM (2.8G and 16.7G). This action of GLP-1 is illustrated on a compressed time scale (A1) that depicts first and second phase GSIS or on an expanded time scale that depicts first-phase GSIS only (A2). Individual values are mean ± SEM. The action of GLP-1 was quantified by AUC analysis for first-phase (B1), second-phase (B2), and total (B3) sum of first and second phases) insulin secretion. Note that the statistically significant threshold for GLP-1 agonist action was ca. 100 pM when monitoring first-phase GSIS (B1; p value 0.0168), whereas it was 1 nM when monitoring second-phase and total GSIS (B2 and B3; p values 0.0095 and 0.0176, respectively). The findings in panels A1–B3 are averaged data with ANOVA analysis obtained from nine independent experiments. The significance (p values) is indicated with accompanying comparisons. Each symbol in the box and whiskers plots is the AUC value obtained when monitoring GSIS from the islets of a single perfusion chamber. C1 and C2, GLP-1R antagonist Ex[9–39] tested at 1 μM blocked the action of 1 nM GLP-1 to potentiate GSIS, as measured in the assays of total GSIS (C1) or first-phase GSIS (C2). The findings presented in panels C1 and C2 are averaged data from five independent experiments. AUC, area-under-the-curve; n.s., not significant.
effect to potentiate GSIS (Fig. 4, A1, A2 and B1–B3). The magnitude of this effect of glucagon was comparable to that measured in response to GLP-1 (cf., Fig. 1, A1 and A2 versus Fig. 4, A1 and A2). Furthermore, as for GLP-1, the potentiation of GSIS by glucagon was blocked by Rp-8-Br-cAMPS-pAB (Fig. 4, C1 and C2).

In comparison with GLP-1, the glucagon concentration-response relationship was right-shifted by ca. 30-fold (cf., Fig. 1, A1 and A2 versus Fig. 4, A1 and A2). This is likely to be explained by the presence of intra-islet glucagon which exerts prior occupancy of the GcgR so that stimulatory effects of 0.3 to 3 nM glucagon are not measurable. In fact, conditioned medium (CM) obtained from rat islet cultures (300 islets in 4 ml media and 20 h exposure) contained glucagon that was bioactive in assays of GcgR agonism. This was established in FRET assays using HEK293 cells expressing the GcgR and H188. Under control conditions, glucagon (0.1–100 nM) increased the levels of cAMP, thereby allowing live-cell calibration of its concentration-response relationship (Fig. 5A1). Based on this calibration, rat islet CM contained ca. 1 nM bioactive glucagon (Fig. 5A2). No effect of CM was measured in HEK293 cells not expressing the GcgR (data not shown). As expected, medium that was not conditioned had no effect (Fig. 5A2). Furthermore, CM obtained from INS-1 832/13 cell cultures was without GcgR-agonist activity (Fig. 5B1), as expected for cells that are of β-cell origin. Using ELISA, it was determined that rat islet CM contained 1061 ± 56 pM glucagon, but only 6 pM glucagon for INS-1 832/13 CM (Fig. 5C1).

We also evaluated the possible presence of GLP-1 in the CM of rat islets. FRET assays using HEK293 cells expressing the GLP-1R demonstrated a stimulatory action of CM to raise levels of cAMP (Fig. 5B2). However, interpretation of this finding is complicated by the fact that GLP-1 and glucagon
both stimulate the GLP-1R. This fact was established in FRET assays that monitor glucagon action at the GcgR (Fig. 5, D1 and D2), or GLP-1 action at the GLP-1R (Fig. 5, E1 and E2), or glucagon action at the GLP-1R (Fig. 5, F1 and F2). Thus, this FRET assay is unable to distinguish between GLP-1 or glucagon in the CM. Therefore, a direct measurement of GLP-1 was performed using ELISA of CM. For rat islets, the CM contained especially low concentrations of GLP-1 (7–13 pM), as was also the case for INS-1 832/13 CM (1 pM) (Fig. 5, C1 and C2).

Although it is impossible to measure the true concentration of intra-islet GLP-1, these findings using rat islet CM are significant in that they offer a simple explanation concerning why administered GLP-1, but not glucagon, acts with high potency to potentiate GSIS. Specifically, if intra-islet concentrations of endogenous GLP-1 are low, there will be little prior occupancy of the β-cell GLP-1R when testing administered GLP-1. Still, a “spillover” effect of intra-islet glucagon will exist at the GLP-1R, thereby resulting in partial occupancy of these receptors. Thus, the apparent potency of GLP-1 in assays of GSIS (Fig. 1, A1, A2 and B1–B3) is less than what is measured when monitoring levels of cAMP in HEK293-H188 GLP-1R cells (Fig. 5, E1 and E2).

Contrasting with the situation described above for GLP-1, the presence of intra-islet glucagon is expected to result in significant prior occupancy of the β-cell GcgR. Thus, the potency of administered glucagon in assays of GSIS is much less than what is measured when monitoring levels of cAMP in HEK293-H188 GcgR cells (Fig. 5, D1 and D2). This phenomenon leads to a right-shift of the concentration-response relationship for glucagon in assays of GSIS, thereby explaining the failure of low concentrations of glucagon to potentiate GSIS (Fig. 4, A1, A2 and B1–B3).

First-phase GSIS stimulated by glucose alone is disrupted by GcgR and GLP-1R antagonists

Intra-islet glucagon released from the islet α-cells might serve as a paracrine hormone that enables adjacent β-cells to exhibit first and/or second phase GSIS. This hypothesis was tested in studies of GSIS under conditions in which islets were stimulated with glucose, while also being treated with the GRA monoclonal antibody LY2786890 or the GLP-1R antagonist Ex[9–39]. Accompanying FRET assays monitoring levels of cAMP validated the specificities with which these antagonists acted at the rat GcgR or GLP-1R.

For islets treated with the GRA (70 nM), a disruption of first-phase GSIS in response to glucose alone was measured, whereas second-phase GSIS was not significantly affected (Fig. 6, A1 and A2). Thus, intra-islet glucagon acting at the
β-cell GcgR is of critical importance to the generation of first-phase GSIS that is cAMP-dependent (25). In marked contrast, the action of administered glucagon (10 nM) to potentiate first and second phase GSIS was not blocked by the GRA (Fig. 6, B1 and B2). These findings are summarized in the accompanying AUC analysis (Fig. 6, C1–C3). Thus, intra-islet glucagon engages the β-cell GcgR to enable first-phase insulin secretion in response to glucose alone, whereas a high concentration of administered glucagon acts independent of the GcgR to potentiate GSIS.

GRA antagonist action was specific for the GcgR without any measurable action at the GLP-1R. This was established in FRET assays monitoring levels of cAMP in HEK293-H188C24 cells stably expressing H188 and transiently transfected with the rat GcgR or GLP-1R. Thus, the GRA (3–1000 nM) blocked the cAMP-elevating action of glucagon (10 nM) at the GcgR (Fig. 6, D1 and D2). In contrast, the GRA failed to block glucagon agonist action at the GLP-1R (Fig. 6E1) or GLP-1 agonist action at the GLP-1R (Fig. 6E2). Furthermore, the GRA tested alone failed to alter levels of cAMP in cells expressing the GcgR or GLP-1R (Fig. 6, F1 and F2). Finally, potentiation of first and second phase GSIS by GLP-1 was not disrupted by the GRA (Fig. S3).

Since first-phase insulin secretion in response to glucose alone is conditional on cAMP signaling, a threshold effect may exist in which levels of β-cell cAMP must exceed some minimal baseline value to support exocytosis. Potentially, intra-islet glucagon acts not only at the GcgR, but also at the GLP-1R to achieve this baseline value. Support for this concept is provided by our finding that first-phase GSIS in response to glucose alone was suppressed by Ex[9–39]. Using methods analogous to those used in studies of the GRA, it was demonstrated that Ex[9–39] (3 μM) suppressed first-phase GSIS in response to glucose, while also exerting a minor inhibitory action to reduce the second-phase GSIS (Fig. 7, A1 and A2). Of major significance, Ex[9–39] also blocked the potentiation of GSIS by 10 nM glucagon (Fig. 7, B1 and B2). This finding demonstrates that a high concentration of

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**Figure 4. Concentration-response relationship for potentiation of GSIS by glucagon in rat islets.** A1 and A2, glucagon potentiated GSIS that was initiated by a step-wise increase of glucose concentration from 2.8 to 16.7 mM. This action of glucagon is illustrated on a compressed time scale (A1) that depicts first and second phase GSIS or on an expanded time scale that depicts first-phase GSIS only (A2). The action of glucagon was quantified by AUC analysis in which the concentration-dependent action of glucagon is depicted in box and whiskers format for first-phase (B1), second-phase (B2), and total (B3) insulin secretion. Note that the statistically significant threshold for glucagon agonist action was ca. 10 nM when monitoring first-phase (B1, p value 0.0001), second-phase (B2, p value 0.0001), and total (B3, p value 0.0001) GSIS. The findings in panels A1–B3 are averaged data with ANOVA analysis obtained from nine independent experiments. Significance (p values) is indicated with accompanying comparisons. Each symbol in the box and whiskers plots is the AUC value obtained when monitoring GSIS from islets of a single perfusion chamber. C1 and C2, Rp-8-Br-cAMPS-pAB tested at 10 μM blocked the potentiation of GSIS by 10 nM glucagon. Representative example from three independent experiments.
Glucagon effectively engages the β-cell GLP-1R. These data are summarized in the accompanying AUC analysis (Fig. 7, \( C_1 \)–\( C_3 \)). It is important to note that the findings summarized above are not explained by an off-target action of Ex\([9–39]\) at the GcgR. In HEK293-H188 C24 cells expressing the rat GcgR, the action of glucagon to raise levels of cAMP was not blocked by Ex\([9–39]\) (Fig. 7\( D_1 \)) nor did Ex\([9–39]\) exert any effect when tested alone (Fig. 7\( D_2 \)). However, Ex\([9–39]\) blocked the cAMP-elevating actions of glucagon and GLP-1 in HEK293-H188 C24 cells expressing the rat GLP-1R (Fig. 7, \( E_1 \) and \( E_2 \)).

A novel prediction derived from this data set is that additive actions of the GRA and Ex\([9–39]\) will not be measurable in assays of first-phase GSIS when these antagonists are tested at saturating concentrations. This prediction is based on the concept that a saturating concentration of each antagonist alone will reduce the levels of cAMP below the minimal threshold value required to allow first-phase GSIS. To test this prediction, the GRA (70 nM) and Ex\([9–39]\) (3 \( \mu \)M) were administered together, while also evaluating the action of glucagon (10 nM) to potentiate GSIS. These experiments demonstrated that dual treatment with the GRA and Ex\([9–39]\) did not reduce first-phase GSIS to a greater extent than what was measured when each was administered alone. Thus, combined treatment with the GRA and Ex\([9–39]\) led to a strong suppression of first-phase GSIS (Fig. 8, \( A_1 \) and \( A_2 \)), but the magnitude of this effect was no greater than what was observed when testing the GRA alone (Fig. 6, \( A_1 \) and \( A_2 \)) or Ex\([9–39]\) alone (Fig. 7, \( A_1 \) and \( A_2 \)). Furthermore, dual treatment...
with the GRA and Ex[9–39] blocked the potentiation of GSIS by glucagon to the same extent as measured when Ex[9–39] was tested alone (Fig. 8, B1 and B2). This finding is expected because Ex[9–39] alone fully blocked the action of glucagon in this assay (Fig. 7, B1 and B2). Accompanying AUC analysis summarizes these findings when testing the GRA, Ex[9–39], and glucagon in assays of first-phase, second-phase, and total GSIS (Fig. 8, C1–C3).

Although GRA and Ex[9–39] antagonist action is readily studied in HEK293-H188 C24 cells that express the recombinant GcgR and GLP-1R (Figs. 6 and 7), a more physiological approach is to evaluate antagonist action in INS-1 832/13 cells that coexpress endogenous GcgR and GLP-1R (41). Unlike rat islets, these cells do not secrete significant quantities of glucagon (Fig. 5), and they are especially sensitive to glucagon acting in FRET assays that monitor levels of cAMP. Thus, glucagon (0.1–30 nM) raised the levels of cAMP in INS-1 832/13 cells, an action reduced but not fully blocked by the GRA (Fig. 8, D1 and D2). This is a novel finding in view of the fact that low concentrations of glucagon failed to exert a stimulatory action in assays of rat islet GSIS (Fig. 4). Thus, the concentration of glucagon in the CM of INS-1 832/13 cells (6 pM) is sufficiently low to allow detection of glucagon agonist action at the GcgR when tested at low concentrations (0.1–3 nM). It is also interesting to note that glucagon action in INS-1 832/13 cells was less sensitive to antagonism by Ex[9–39] in comparison with findings obtained in assays of rat islet GSIS (Fig. 8E1). Importantly, neither the GRA nor Ex[9–39] altered the levels of cAMP on their own (Fig. 8E2). Finally, a dual agonist action of glucagon was established by demonstrating additive actions
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Figure 7. Ex[9–39] inhibits first-phase GSIS in response to glucose alone, while also blocking the potentiation of GSIS by glucagon. A₁ and A₂, Ex[9–39] (3 μM) inhibited first-phase GSIS in response to 16.7 mM glucose alone. A minor inhibitory action of Ex[9–39] to inhibit second-phase GSIS was also measurable. B₁ and B₂, Ex[9–39] (3 μM) blocked the potentiation of first and second phase GSIS by glucagon (10 nM). C₁–C₃, AUC analysis of Ex[9–39] antagonist action to alter first-phase, second-phase, or total insulin secretion using experimental designs shown in A₁–B₂. The islets were exposed to 16.7 mM glucose in the presence or absence of glucagon, with or without Ex[9–39]. The findings in A₁–C₃ are averaged data analyzed by ANOVA and obtained from three independent identical experiments. Significance (p values) is indicated with accompanying comparisons. Each symbol in the box and whiskers plots is the AUC value obtained when monitoring GSIS from islets of a single perfusion chamber. D₁ and D₂, Ex[9–39] failed to block the action of glucagon to raise levels of cAMP (D₁), while also having no effect on its own (D₂) in HEK293-H188 c24 cells transfected with the rat GcgR. E₁ and E₂, Ex[9–39] blocked the action of glucagon (IC₅₀ 50 nM) to raise levels of cAMP in HEK293-H188 c24 cells transfected with the rat GLP-1R. F₁ and F₂, Ex[9–39] blocked the action of GLP-1 (IC₅₀ 18 nM) to raise levels of cAMP in HEK293-H188 c24 cells transfected with the rat GLP-1R.

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of the GRA and Ex[9–39] to suppress cAMP production (Fig. 8, F₁ and F₂).

GcgR and GLP-1R antagonists fail to reduce GSIS stimulated by a glucose gradient ramp

Oral administration of glucose in an OGTT leads to a slow increase of blood glucose concentration rather than the sudden step-wise increase used to monitor first and second phase GSIS (5, 45). Because this gradual increase of blood glucose levels is a more physiological stimulus for insulin secretion (55), it was of interest to test if intra-islet glucagon regulates GSIS that is measurable in vitro using islets exposed to a gradient of slowly increasing concentrations of glucose.

Monophasic insulin secretion from rat islets was measured in response to a linear gradient of glucose concentrations beginning at 3 mM and ending at 30 mM when delivered over a 50 min time course. Unlike its inhibitory effect in step-wise assays of first-phase GSIS, the GRA failed to alter insulin secretion stimulated by a glucose gradient alone (Fig. 9, A₁–A₃). Independent confirmation of this finding was obtained using the GcgR antagonist des-His¹-Glu⁹-glucagon (Fig. S4). Similarly, Ex[9–39] also failed to alter insulin secretion in response to a glucose gradient alone (Fig. 9, B₁–B₃). Thus, GSIS stimulated by a glucose gradient is not conditional on intra-islet glucagon acting at the GcgR or GLP-1R. Still, glucagon (10 nM) potentiated GSIS in the gradient assay, an action insensitive to the GRA (Fig. 9, A₁–A₃), but blocked by Ex[9–39] (Fig. 9, B₁–B₃). Furthermore, the GRA failed to block the potentiation of insulin secretion by GLP-1 (1 nM), whereas Ex[9–39] was effective (Fig. S5). Thus, glucagon and GLP-1 exerted their stimulatory effects solely through the GLP-1R.
Overall, this pharmacological profile for GcgR and GLP-1R agonist or antagonist action in glucose gradient assays is notable in that it reproduces findings obtained in studies of second-phase GSIS (cf., Figs. 6–8). Potentially, GSIS stimulated by the glucose gradient recruits a mechanism of cAMP-independent insulin secretion that is shared by second-phase GSIS and that is largely insensitive to intra-islet glucagon. In fact, Rp-8-Br-cAMPS-pAB failed to inhibit GSIS measured in the gradient assay, although it blocked the potentiation of GSIS by glucagon (Fig. 9, C1–C3).

**Discussion**

**Intra-islet glucagon modifies the potency and receptor selectivity of administered glucagon**

Here, we applied automated methods of islet perfusion in combination with fast sample rates to monitor the kinetics of insulin secretion so that it would be possible to determine whether intra-islet glucagon acts as a competence factor to support first and/or second phase GSIS. An additional goal was to test a hypothetical receptor occupancy model of GPCR agonist action in which the presence of intra-islet glucagon modifies GcgR and/or GLP-1R agonist potency and selectivity. We also sought to establish the relative importance of cAMP signaling to the genesis of first and second phase GSIS under conditions in which islets were stimulated with glucose alone or glucose in combination with GcgR and GLP-1R agonists. Complementary FRET assays using cAMP biosensors allowed the receptor selectivities of tested compounds to be validated using HEK293 cells expressing recombinant GcgR and GLP-1R or INS-1 832/13 cells that coexpress endogenous GcgR and GLP-1R.

As depicted in the summary model (Fig. 10), these approaches revealed that first-phase GSIS was conditional on a...
paracrine hormone action of intra-islet glucagon. Although it was not technically possible to measure true intra-islet levels of glucagon, it was instead possible to measure glucagon in the CM of islet cultures. This approach allowed an estimation of the relative concentrations of glucagon and GLP-1 that might exist within the islet interstitium. Based on EC50 values for glucagon and GLP-1 obtained in FRET assays that detect cAMP, it is now possible to construct a hypothetical receptor occupancy model (Fig. 10) that seeks to explain how intra-islet glucagon alters the actions of administered glucagon and...
GLP-1 when each is tested in assays of GSIS. As validated in our FRET assays using H188 and AKAR3, this model assumes that glucagon binds with high affinity to the GcgR but low affinity to the GLP-1R. It also assumes high affinity binding of GLP-1 to the GLP-1R, with no binding to the GcgR.

When neither glucagon nor GLP-1 is administered (Fig. 10A1), intra-islet glucagon occupies a higher proportion of GcgR (red) in comparison with GLP-1R (blue). Thus, the GRA blocks first-phase GSIS in response to glucose alone (Fig. 10A2). When intra-islet glucagon is paired with a high concentration of administered glucagon (Fig. 10B1), additional GLP-1R are recruited so that GLP-1R-stimulated GSIS predominates. Thus, first-phase GSIS is not sensitive to the GRA and is instead blocked by Ex[9–39] (Fig. 10B2). Finally, when intra-islet glucagon is paired with a saturating concentration of administered GLP-1 (Fig. 10C1), there is full occupancy of the GLP-1R, and under these conditions Ex[9–39] but not the GRA blocks first-phase GSIS (Fig. 10C2).

One additional prediction of this model is that prior occupancy of the GcgR by intra-islet glucagon explains why low concentrations (0.1–3 nM) of administered glucagon are ineffective in assays of GSIS. In fact, the assays reported here used islets pre-equilibrated in 2.8 mM glucose, a concentration that stimulates α-cell glucagon secretion (Fig. S6). This intra-islet endogenous glucagon competes with administered exogenous glucagon for the GcgR, and if concentrations of both are similar, it is not possible to measure a stimulatory action of administered glucagon (Fig. 10D1). When the concentration of administered glucagon is increased to 10 nM, glucagon achieves its capacity to signal through the GLP-1R (Fig. 10D2). The net effect is a major right-shift of the glucagon concentration-response relationship when comparing its ability to stimulate insulin secretion from islets, versus its ability to stimulate cAMP production in HEK293 GcgR and GLP-1R cell lines that do not secrete glucagon. Note that an especially low concentration of intra-islet GLP-1, as inferred from ELISA of...
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In response to intra-islet glucagon is not sufficiently strong to upregulate cAMP-dependent exocytosis during second-phase GSIS. In contrast, there is a strong upregulation of second-phase GSIS that is Rp-8-Br-cAMPS-pAB sensitive when the islets are treated with exogenous glucagon or GLP-1. These novel findings indicate that high levels of exogenous glucagon engage the GLP-1R to strongly stimulate cAMP production and to allow cAMP-dependent exocytosis to become operational during second-phase GSIS.

Identifying the mechanism for this switch from cAMP-independent to cAMP-dependent insulin secretion is of great interest. Shibasaki et al. (33) reported a cAMP-regulated mechanism of insulin secretion that results from “restless newcomer” exocytosis in which Epac2 activation mobilizes cytoplasmic secretory granules so that they quickly transit to the plasma membrane for release without pausing in a “docked” state. When islets are stimulated with glucose alone, this newcomer exocytosis is more prominent during first-phase as compared with second-phase GSIS. Raising levels of cAMP by treatment with forskolin potentiates newcomer exocytosis during first-phase, while also greatly increasing newcomer exocytosis during second-phase (33). Based on current concepts of cytosolic cAMP “microdomains” (70), we propose the novel hypothesis that first but not second phase newcomer exocytosis is stimulated by spatially restricted cAMP generated by intra-islet glucagon acting at the GcgR. In contrast, exogenous glucagon, acting through the GLP-1R, expands these microdomains to recruit additional newcomer exocytosis during second-phase GSIS.

Contrasting roles for intra-islet glucagon in assays of monophasic versus biphasic GSIS

An additional novel discovery reported here is that GcgR and GLP-1R antagonists fail to inhibit insulin secretion measured in response to an imposed linear increase of the glucose concentration. We interpret this finding to indicate that monophasic insulin secretion measured in the glucose gradient assay is not under the control of intra-islet glucagon. This concept is supported by our finding that monophasic insulin secretion is insensitive to the cAMP antagonist Rp-8-Br-cAMPS-pAB. Still, monophasic insulin secretion is strongly potentiated by glucagon, an action blocked by GLP-1R antagonist Ex[9–39] but not the GcgR antagonists LY2786890 or des-His<sup>1</sup>-Glu<sup>9</sup>-glucagon. Thus, monophasic insulin secretion in response to glucose alone, or in response to glucose paired with glucagon, exhibits pharmacological features similar to second-phase GSIS measured in step-wise assays of biphasic insulin secretion. These surprising findings suggest that under conditions in which glucose is administered by itself, monophasic and second-phase insulin secretion share a common mechanism of cAMP-independent insulin exocytosis that is not contingent on intra-islet glucagon acting at the GcgR or GLP-1R. Instead, our studies using the step-wise assay reveal that the action of intra-islet glucagon is specific for the first-phase GSIS that is cAMP-dependent. This discovery is made possible by our use of rat

Glucagon and GLP-1 enable cAMP-dependent exocytosis during second-phase GSIS

Studies reported here are novel in that they expand upon our prior finding that first-phase but not second-phase insulin secretion in response to glucose alone is blocked by Rp-8-Br-cAMPS-pAB (25). We find that first-phase GSIS is entirely cAMP-dependent, and that this cAMP is generated by intra-islet glucagon acting with high affinity at the β-cell GcgR. A low affinity spillover effect of glucagon to stimulate the GLP-1R is also measurable. Because second-phase GSIS is relatively resistant to Rp-8-Br-cAMPS-pAB, we propose that β-cell cAMP production islet CM, will not lead to a major right shift of the GLP-1 concentration-response relationship (Fig. 10D<sub>2</sub>). Thus, the threshold for potentiation of GSIS by administered GLP-1 is ca. 100 pM, a value closer to that measured in FRET assays using HEK293-GLP-1R cells that do not secrete GLP-1.

Does intra-islet GLP-1 contribute to β-cell glucose competence?

The concept of β-cell glucose competence was first advanced based on the abilities of glucagon or GLP-1 to restore the capacity of rat β-cells to respond to glucose under conditions of primary cell culture. This restorative effect was measurable in assays of insulin secretion (56–58), and in patch clamp assays that monitor the ability of glucose to promote the closure of K<sub>ATP</sub> channels (59, 60). Thus, the question arises as to whether intra-islet GLP-1 serves as a paracrine hormone important to β-cell glucose competence. α-cells synthesize proglucagon that is the precursor for glucagon and possibly GLP-1 under conditions of stress (61–66). Yet for rat islets, we found no evidence for significant levels of GLP-1, a finding consistent with prior reports using human, mouse, and pig islets (67, 68). This was demonstrated by the immunooassay of rat islet CM using anti-GLP-1 antibodies that detect total GLP-1 measured in this manner ranged from 7 to 13 pM, concentrations that are too low to stimulate insulin secretion from rat islets. Furthermore, we were unable to substantiate reports that extremely low concentrations of GLP-1 (0.1–10 pM) stimulate insulin secretion from mouse islets (49–51). In contrast, the levels of glucagon in rat islet CM were high (1 nM), thereby indicating that glucagon is the primary intra-islet hormone important to β-cell glucose competence under conditions of islet isolation. Interestingly, we obtained the novel finding that Prodo PIM(S) is useful for stabilization of glucagon so that it can be measured after freeze-thawing, while also retaining its agonist activity at the GcgR. This is noteworthy in view of the fact that Prodo PIM(S) is the optimal medium for primary culture of human islets (69). Thus, glucagon secreted from human islets may accumulate in culture medium in its fully bioactive form, thereby allowing it to enhance β-cell functionality.

Glucagon and GLP-1 enable cAMP-dependent exocytosis during second-phase GSIS

An additional novel discovery reported here is that GcgR and GLP-1R antagonists fail to inhibit insulin secretion measured in response to an imposed linear increase of the glucose concentration. We interpret this finding to indicate that monophasic insulin secretion measured in the glucose gradient assay is not under the control of intra-islet glucagon. This concept is supported by our finding that monophasic insulin secretion is insensitive to the cAMP antagonist Rp-8-Br-cAMPS-pAB. Still, monophasic insulin secretion is strongly potentiated by glucagon, an action blocked by GLP-1R antagonist Ex[9–39] but not the GcgR antagonists LY2786890 or des-His<sup>1</sup>-Glu<sup>9</sup>-glucagon. Thus, monophasic insulin secretion in response to glucose alone, or in response to glucose paired with glucagon, exhibits pharmacological features similar to second-phase GSIS measured in step-wise assays of biphasic insulin secretion. These surprising findings suggest that under conditions in which glucose is administered by itself, monophasic and second-phase insulin secretion share a common mechanism of cAMP-independent insulin exocytosis that is not contingent on intra-islet glucagon acting at the GcgR or GLP-1R. Instead, our studies using the step-wise assay reveal that the action of intra-islet glucagon is specific for the first-phase GSIS that is cAMP-dependent. This discovery is made possible by our use of rat
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islets as the test system. Unlike mouse islets that were used in prior studies of intra-islet glucagon action (15, 16), rat islets exhibit substantial second-phase GSIS that is easily distinguishable from first-phase on the basis of kinetics (71). Because a gradual rather than step-wise increase of blood glucose is the true stimulus for insulin secretion under conditions of oral glucose administration, it might be that intra-islet glucagon plays a less significant role as a determinant of outcomes obtained in an OGTT. Instead, a more important role for intra-islet glucagon is expected when evaluating outcomes obtained in the intravenous glucose tolerance test since infusion of glucose leads to a rapid increase of blood glucose levels and first-phase GSIS.

Potential physiological relevance

An important question concerns whether nonphysiological accumulation of glucagon within the interstitial space of isolated islets leads to an “apparent” paracrine hormone effect that does not occur in vivo. In fact, microvascular blood flow through the islets may efficiently clear glucagon from the interstitial space. If so, our receptor occupancy model (Fig. 10) predicts that GgCR and GLP-1R antagonists will fail to inhibit insulin secretion in vivo in response to glucose alone. This prediction is consistent with the findings of Moens et al. (11) using a rat perfused pancreas model in which perfusion emulates the effect of blood flow to clear glucagon from the islet’s interstitial space. In fact, Moens et al. (11) reported that insulin secretion in response to glucose alone was not reduced by GgCR antagonist des-His1-Glu9-glucagon or GLP-1R antagonist Ex[9–39]. In marked contrast, Svendsen et al. (14) used a perfused mouse pancreas model in which Ex[9–39] reduced insulin secretion in response to glucose alone, although a GgCR antagonist was not tested. The reason for this discrepancy is unknown but might reflect a species difference.

Our receptor occupancy model predicts that low concentrations of administered glucagon will act in vivo at the β-cell GgCR to stimulate insulin secretion provided that intra-islet glucagon is already cleared from the interstitial space. In fact, Moens et al. (11) reported that for perfused rat pancreas, 1 nM glucagon stimulated insulin secretion. Similarly, in studies of perfused mouse pancreas, Svendsen et al. (14) reported stimulation of insulin secretion by 0.1 to 10 nM glucagon, an effect mediated by both the GgCR and GLP-1R. Additional studies using genetically engineered mice provide support for in vivo paracrine actions of glucagon that are mediated by the GgCR and/or GLP-1R (15, 16). However, compensatory changes of hormone and receptor gene expression in these model systems may cloud the interpretation of findings (72, 73). Thus, the relevance of glucagon acting as an intra-islet paracrine hormone at the GgCR and/or GLP-1R in vivo has yet to be fully validated.

Advantages and limitations of this study

The interpretation of findings reported here for intra-islet control of GSIS by glucagon is based on our use of LY2786890, a monoclonal antibody GgCR antagonist that does not exhibit partial agonist or inverse agonist actions in assays of cAMP, and that does not exert an off-target action at the GLP-1R (41). Although GgCR antagonist MK0893 was recently reported to inhibit glucagon-stimulated insulin secretion from mouse islets (74), it is an allosteric inhibitor that not only antagonizes glucagon action at the GgCR but also the GLP-1R (41). Thus, our studies using LY2786890 and isolated islets more clearly substantiate an ability of intra-islet glucagon to confer β-cell glucose competence for first-phase GSIS in a GgCR-mediated manner. Finally, controversy exists concerning whether Ex[9–39] acts as a pure antagonist or instead as an inverse agonist at the GLP-1R (14, 43, 75). If Ex[9–39] possesses such inverse agonist properties, it may directly block first-phase insulin secretion in a manner that is independent of any action of intra-islet glucagon at the GLP-1R.

Conclusion

Prior efforts in T2D therapeutics focused on achieving functional restoration of first and second phase GSIS by administered GLP-1R agonists such as exenatide (76). The findings presented here provide a new mechanistic explanation for this beneficial effect. GLP-1R stimulation by GLP-1 leads not simply to a potentiation of first-phase GSIS, but also a recruitment of cAMP-dependent second-phase GSIS that is missing in the absence of GLP-1. Remarkably, high concentrations of glucagon engage the GLP-1R to achieve a similar effect. Increasingly, it is appreciated that glucagon acting at the GgCR and GLP-1R plays an important role in systemic glucose homeostasis (77). Collectively, we expect that new findings reported here will advance T2D drug discovery in that they emphasize the likely clinical relevance of synthetic dual agonist peptides that simultaneously stimulate the GgCR and GLP-1R (78).

Experimental procedures

Cell culture

INS-1 832/13 cells were a gift from C. Newgard and were grown in RPMI-1640 culture medium for passaging using the original protocol of Hohmeier et al. (46). The parental HEK293 cell line was obtained from the American Type Culture Collection. HEK293 cells stably expressing the rat GgCR (Fig. 5, A1, A2 and B1) at a density of ca. 250,000 receptors/cell were obtained from T.P. Sakmar (79). HEK293 cells stably expressing the human GLP-1R (Fig. 5B2) at a density of ca. 150,000 receptors/cell were obtained from Novo Nordisk A/S (80). HEK239-H188 c24 cells stably expressing H188 (Figs. 5, D1–F2, 6, D1–F2 and 8, D1–F2) were generated by O.G. Chepurny in the Holz laboratory (81). All HEK293 cell cultures were maintained in DMEM containing 25 mM glucose and supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cell cultures equilibrated at 37 °C in a humidified incubator that was gassed with 5% CO2 were passaged once a week. Culture media and additives were obtained from Thermo Fisher Scientific.
Cell transfection

HEK293-H188 c24 cells stably expressing H188 were obtained by G418 antibiotic resistance selection using our published methods (81). Transient transfections of HEK293-H188 c24 cells for the expression of GPCRs were performed with Lipofectamine and Plus Reagent (Thermo Fisher) using our published methods (81). Plasmids containing the rat GcgR cDNA (79) or human GLP-1R cDNA (82) were provided by T.P. Sakmar and M. Beinborn, respectively. Adenoviruses for transduction of HEK293 cells were generated by a commercial vendor (ViraQuest) using the shuttle vector pVQAd CMV K-NpA and the H188 plasmid provided by Kees Jalink (47) or the AKAR3 plasmid provided by Jin Zhang (48).

FRET reporter assay in a 96-well format

HEK293 cells stably expressing recombinant GPCRs were plated at 80% confluence on 96-well clear-bottom assay plates (Costar 3904, Corning) coated with rat tail collagen (Collaborative Biomedical Products). The cells were then transduced for 16 h with H188 virus at a density of ca. 60,000 cells/well under conditions in which the multiplicity of infection was equivalent to 25 viral particles per cell. The culture media was removed and replaced by 200 μl/well of a standard extracellular saline (SES) solution supplemented with 11 mM glucose and 0.1% bovine serum albumin. The composition of the SES was (in mM): 138 NaCl, 5.6 KCl, 2.6 CaCl2, 1.2 MgCl2, 11.1 glucose, and 10 Hepes (295 mOsmol, pH 7.4). Real-time kinetic assays of FRET were performed using a FlexStation 3 microplate reader equipped with excitation and emission light monochromators (Molecular Devices) (41). Excitation light was delivered at 435/9 nm (455 nm cut-off), and emitted light was detected at 485/535 nm (CFP) or 535/15 nm (YFP). The emission intensities were the averages of 12 excitation flashes for each time point per well. Test solutions dissolved in SES were placed in V-bottom 96-well plates (Greiner Bio-One), and an automated pipetting procedure was used to transfer 50 μl of each test solution to each well of the assay plate containing monolayers of these cells. The 485/535 emission ratio was calculated for each well and the mean ± SEM values for 12 wells were averaged. These FRET ratio values were normalized using baseline subtraction so that the y-axis value of 0 corresponds to the initial baseline FRET ratio, whereas a value of 100 corresponds to a 100% increase (i.e., doubling) of the FRET ratio. The time course of the ΔFRET ratio was plotted after exporting data to Origin data analysis software (OriginLab). Origin was also used for nonlinear regression analysis to quantify dose-response relationships.

Perfusion assays of rat islet GSIS

Sprague-Dawley rats obtained from Envigo RMS were fed a standard chow diet (TekLad Diet 2014; Harlan Laboratories) and were housed in an AAALC accredited vivarium at Lilly Research Laboratories. For 12-week-old male rats, the pancreas was surgically removed under conditions of isoflurane anesthesia after cervical dislocation, as stipulated in an animal use protocol approved by the Eli Lilly Institutional Animal Care and Use Committee. After inflation of the pancreas with a Hank’s balanced salt solution (Life Technologies, Cat. No. 14175–103) containing collagenase (VitaCye, LCC; Cat. No. 005–1030), the pancreas was subjected to collagenase digestion (14 min) to obtain islets. These islets were cultured overnight in RPMI-1640 medium containing 11.1 mM glucose, 10% fetal bovine serum, glutamine (2 mM GlutaMax; Life Technologies), and penicillin-streptomycin. Perfusion assays of secreted insulin were performed the next day. Briefly, 50 islets were immobilized on a 24 well matrix (Bio-Gel, Bio-Rad Laboratories) within individual perfusion chambers housed in a 37 °C climate-controlled enclosure so that automated delivery of test solutions at a flow rate of 100 μl/min could be achieved using a Biorep Technologies Perfusion System. Thus, for each chamber, a peristaltic pump delivered Hepes-buffered saline solution containing (mM): 120 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgCl2, 10 Hepes, 24 Na2HCO3, and 0.25% BSA. The perfusate samples were collected at 4 °C using a robotic fraction collector (Biorep Tech.) designed for 96-well plates. Insulin content of the perfusates was quantified by electrochemical luminescence detection using an MSD Insulin Assay Kit (Meso Scale Diagnostics; Cat. No. K152BZC). The amount of secreted insulin present within each perfusate sample was normalized relative to islet DNA content for each chamber, as determined using a MagMax-96 DNA assay kit (Life Technologies; Cat. No. 4413021).

Static incubation assays of mouse islet GSIS

C57BL/6J mice were obtained from Envigo RMS. Diet, housing, animal approval protocols, methods of islet isolation, and culture media were as described above for rats. Static incubation assays of GSIS were performed using freshly isolated islets after 2 h in culture or using islets cultured overnight for 24 h. Briefly, three islets were hand-picked into each well of a 48-well tissue culture plate containing 150 μl Hepes-buffered saline solution containing (mM): 120 NaCl, 4.8 KCl, 2.5 CaCl2, 1.2 MgCl2, 10 Hepes, 24 Na2HCO3, 2.8 glucose, and 0.25% BSA. An additional 150 μl solution was added so that a y-axis value of 1 corresponds to a 100% increase in insulin concentration (i.e., doubling) of the FRET ratio. The time course of the ΔFRET ratio was plotted after exporting data to GraphPad Prism software.

Detection of glucagon and GLP-1

Detection of glucagon and GLP-1 in CM of rat islet and INS-1 832/13 cell cultures was performed after a 20 h exposure to culture medium. For this analysis, PIM(S) standard islet culture medium (Prodo Lab) was chosen because unlike RPMI-1640, it stabilizes glucagon so that repeated freeze-thawing is possible without the loss of GcgR stimulating properties. For each experiment, 300 rat islets were cultured in 4 ml of media, whereas for INS-1 832/13 cells, 4 ml was obtained from cultures at ca. 80% confluence. For detection of
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glucagon in the media, we used a Mercodia Glucagon ELISA-10 μl kit (Mercodia AB; Cat. No. 10-1281-01). For detection of total GLP-1, we used a Mercodia Total GLP-1 NL-ELISA kit (Cat. No. 10-1278-01) or an MSD V-PLEX kit (Meso Scale Diagnostics; Cat. No. K1503PD).

Sources of reagents

Glucagon, LY2786890, and LY333531 were generated in-house at Lilly, GLP-1[7-36]amide (Cat. No. H-6795) and Ex9–39 (Cat. No. H-8740) were from Bachem. des-His1-Glu2-Glucagon (Cat. No. 11084-95-2) was from Sigma Aldrich. Glucagon (Cat. No. 11084-95-2) was from Sigma Aldrich. 6-Bnz-U73122 (Cat. No. 112648-68-7), U73343 (Cat. No. 142878), Glucagon (Cat. No. 11084-95-2) was from Sigma Aldrich. 6-Bnz-cAMP-AM (Cat. No. B 079) and 8-pCPT-2'-O-Me-cAMP-AM (Cat. No. C 051) were provided by Biolog Life Science Institute GmbH & Co KG. Rp-8-Br-cAMPS-pAB was provided by Biolog Life Science Institute for research purposes.

Administration of test reagents to islets

At the start of each experiment, the GcgR, GLP-1R, and cAMP agonists or antagonists were preadministered to perfused islets as solutions in Hepes-buffered saline containing 2.8 mM glucose for 10 to 15 min. Such conditions favor α-cell glucagon release, while also suppressing β-cell insulin release (Fig. S6). Thus, at 2.8 mM glucose, intra-islet glucagon competes with administered GcgR and GLP-1R antagonists for binding to β-cell glucagon and GLP-1 receptors. These same test reagents were also present when islets were exposed to high concentrations of glucose, but they were absent upon reduction to 2.8 mM glucose at the end of each experiment.

Statistical analyses

GSIS data presented in histogram format are the mean ± SEM. These data were evaluated for statistical significance by a one-way ANOVA test with Dunnett’s multiple comparisons test using GraphPad Prism v.9.1.2, which was also used to construct box and whiskers plots. Comparisons of individual data sets are defined in the accompanying figure legends. A p value of <0.05 was considered to be statistically significant. The box and whisker plots derived from these data show the following: mean (solid square), 25 to 75% range (open box), median (line across open box), and minimum and maximum values (whiskers). FRET data from individual experiments are expressed as the mean ± SEM and are derived from n = 12 wells for each concentration of test agent. The repeatability of findings was confirmed by performing each FRET experiment a minimum of three times.

Data availability

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

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: AUC, area-under-the-curve; BSA, bovine serum albumin; CM, conditioned medium; GcgR, glucagon receptor; GLP-1R, glucagon-like peptide-1 receptor; GRA, GcgR antagonist; GSIS, glucose-stimulated insulin secretion; IVGTT, intravenous glucose tolerance test; OGTT, oral glucose tolerance test; SES, standard extracellular saline; T2D, type 2 diabetes.

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