Pancreatic β cell dysfunction is pathognomonic of type 2 diabetes mellitus (T2DM) and is driven by environmental and genetic factors. β cell responses to glucose and to incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) are altered in the disease state. While rodent β cells act as a coordinated syncytium to drive insulin release, this property is unexplored in human islets. In situ imaging approaches were therefore used to monitor in real time the islet dynamics underlying hormone release. We found that GLP-1 and GIP recruit a highly coordinated subnetwork of β cells that are targeted by lipotoxicity to suppress insulin secretion. Donor BMI was negatively correlated with subpopulation responses to GLP-1, suggesting that this action of incretin contributes to functional β cell mass in vivo. Conversely, exposure of mice to a high-fat diet unveiled a role for incretin in maintaining coordinated islet activity, supporting the existence of species-specific strategies to maintain normoglycemia. These findings demonstrate that β cell connectedness is an inherent property of human islets that is likely to influence incretin-potentiated insulin secretion and may be perturbed by diabetogenic insults to disrupt glucose homeostasis in humans.

Introduction

Type 2 diabetes mellitus (T2DM) currently affects approximately 8.3% of the adult population worldwide, an incidence expected to increase further in the coming years (1). Changes in functional pancreatic β cell mass, the sole source of circulating insulin in vertebrates, are characteristic of this condition and act in concert with defective insulin action to reduce glucose tolerance in genetically susceptible individuals (2). Increased circulating levels of glucose, fatty acids, and other reactive species then drive the complications of this disease including stroke, cardiovascular disease, retinopathy, renal failure, and cancer (3, 4).

The mechanisms underlying glucose-stimulated insulin secretion (GSIS) from single β cells are increasingly well characterized and involve uptake of the sugar via specific glucose transporters (5), enhanced ATP synthesis (6), and the closure of ATP-sensitive K+ channels (KATP) (7). The consequent plasma membrane depolarization leads to Ca2+ influx (8) and exocytosis from secretory granules (9), further potentiated by “KATP-independent” signals (10). In addition to glucose, a range of other secretagogues, including gut-derived incretins, serve to amplify insulin release. Thus, in response to food transit, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) (11) are released by enteroendocrine L and K cells, respectively, from where they augment GSIS to counteract the postprandial hyperglycemic spike (12–15). This so-called “incretin effect” requires elevated glycemia, and GLP-1 and GIP receptor activation engages intracellular signaling mechanisms largely dependent on cyclic adenosine monophosphate (cAMP) generation (16–18) and activated Ca2+ influx (19). Since incretins also stimulate antiapoptotic and prosurvival pathways in β cells (20), GLP-1 mimetics and dipeptidyl peptidase-4 (DPP-4) inhibitors have become first-line antihyperglycemic agents for the treatment of T2DM (21). However, both GLP-1- and GIP-induced insulin secretion are impaired in T2DM (22–25), and patients may respond nonoptimally to both endogenous and exogenous incretin.

Efforts to better understand the interactions that drive GLP-1–augmented (and GIP-augmented) insulin secretion from intact islets have, however, proved challenging due to a lack of uniformity in microorgan architecture between species. This is evident in both the proportions and spatial organization of β, α, and other cell types (26). Phylogenetic differences in insulin secretion may consequently arise from heterogeneity in the intraislet regulation of β cell dynamics, alongside the simple summation of distinct cell biophysical properties. Thus, in humans, the release of insulin from ensembles of stochastically-behaving β cells is likely to reflect their unique cytoarchitectural arrangement along laminar epithelial sheets, combined with alterations to paracrine and neural input (27–30). As such, rodents may represent a nonideal model for investigations of human pancreatic β cell dysfunction, especially if diabetogenic insults perturb species-specific intraislet mechanisms governing β cell population responses.

Using in situ imaging approaches, together with large-scale correlation analyses to map cell-cell interconnectivity (31), we demonstrate that GLP-1 recruits a highly coordinated subnetwork of β cells to augment GSIS, and this is targeted by lipotoxic insults to reduce insulin secretion. Providing evidence for an association with circulating lipid levels in humans, donor BMI was found to be negatively correlated with the coordinated β cell mass, the sole source of circulating insulin in ver-
responses to GLP-1. We believe, therefore, that these results reveal a novel mode of incretin action that may be targeted by known T2DM risk factors to impact insulin release from human islets.

**Results**

GLP-1 activates a coordinated subpopulation of β cells. Multicellular calcium (Ca^{2+}) imaging was used to simultaneously monitor Ca^{2+} signals emanating from fluo-2–loaded β cells residing within the first few layers of intact human islets (Supplemental Figure 1A [supplemental material available online with this article; doi:10.1172/JCI68459DS1]; for data from \( n = 21 \) separate normoglycemic donors, see Supplemental Table 1; donors across the BMI range were selected for experiments using multiple preparations). Since exocytosis in electrically excitable endocrine cells is Ca^{2+} dependent (32), the effects of intervention on the cell population dynamics underlying insulin release were determined by subjecting the resulting traces to large-scale correlation analyses (31). We note that, although α and other neuroendocrine cells comprise a substantial proportion (~40%) of the human islet (26), the former are unlikely to contribute to the recordings here, since elevated glucose is expected to have no effect or to reduce intracellular free Ca^{2+} concentrations ([Ca^{2+}]i) in these cells (33).
Long-lasting optical recordings (30–60 minutes) revealed that, in the presence of elevated (11 mM) glucose, β cells displayed moderate levels of coordinated activity due to stochastic Ca²⁺ oscillations, as previously reported (Figure 1A and refs. 27, 34). Following the application of 10 fM–100 nM (peak response 20 nM) GLP-1 (7-36 human amide; Supplemental Figure 1, B–E), large and synchronous deflections in [Ca²⁺]i were associated with dramatic improvements in coordinated β-cell activity (Figure 1B and C; Supplemental Video 1). This was due to the recruitment of a subpopulation of GLP-1–responsive cells (~56% of glucose-responsive cells, assumed to be β cells, Figure 1D and E) whose levels of correlated Ca²⁺-spiking activity could be dynamically and transiently modified by incretin in a glucose-dependent and GLP-1 receptor–dependent (GLP-1R–dependent) manner (43.6 ± 2.7 versus 92.8 ± 3.7% significantly correlated cell pairs, before and during GLP-1 20 nM application, respectively; n = 9 islets from 4 donors, P < 0.01; Figure 1F and G; Supplemental Figure 2). The 3D tissue context was essential for supporting GLP-1–driven upregulation of coordinated cell activity, since this was reduced to almost control levels (~60%) in dissociated islets (Figure 1H). The residual synchronization likely reflects both the presence of cells (10%–20%) that remained physically connected (Figure 1I) and the random probability of synchronous activity in otherwise unconnected cells.

Gap junctions orchestrate spatiotemporally precise responses to GLP-1. (A) BGA, an inactive analog of the GJ blocker AGA, does not affect coordinated β cell responses to 11 mM glucose (G11) plus GLP-1. Top panel: representative Ca²⁺ traces; red, smoothed; gray, raw. Bottom panel: heatmap depicting minimum (0) to maximum (100) for each cell. (B) As for A, except the application of AGA induces asynchrony in the GLP-1–treated population, which presents as lagged responses to stimulus. (C) Histogram showing the mean percentage of significantly correlated cell pairs in BGA- and AGA-treated islets before (0–200 seconds), during (201–400 seconds), and after (401–1,000 seconds) GLP-1 application (**P < 0.01 versus AGA; two-way ANOVA; n = 8 islets from 3 donors). (D) Probability histograms of distances between correlated β cell pairs in BGA-treated (top panel) and AGA-treated (bottom panel) islets. AGA decreases the distances over which cells coordinate their activity as demonstrated by the left-shifted Gaussian peak (curve fitted to binned data from 3 donors). (E) AGA does not significantly alter the mean percentage of GLP-responsive cells (P = 0.14; Mann-Whitney U test). (F) AGA decreases GLP-1–stimulated insulin release measured in real time using the Zn²⁺-sensitive probe ZIMIR (left panel; mean traces from 3 donors). Bar graph (right panel) showing decreased mean AUC in AGA-treated islets (**P < 0.01; Mann-Whitney U test; n = 7–8 islets from 3 donors). (G) 11 mM glucose-stimulated (G11-stimulated) insulin secretion is identical in AGA– and BGA-treated islets (representative traces from n = 4 recordings).
signaling events between β cells. Homotypic gap junctions (GJs) composed of connexin 36 (Cx36) are present between human and rodent β cells (35, 36) and play an important role in coordinating the cell-cell information exchanges that underlie pulsatile insulin release from rodent islets (36, 37). Application of the GJ blocker, 18-α-glycyrrhetinic acid (AGA), but not its inactive analog β-glycyrrhizic acid (BGA), induced asynchrony in the incretin-sensitive subpopulation (Figure 2, A and B) and significantly reduced the ability of β cells to mount coordinated Ca^{2+} rises in the presence of GLP-1 (83.6 ± 4.4 versus 55.10 ± 7.7% significantly correlated cell pairs at 200 seconds, BGA versus AGA, respectively; n = 8 islets from 3 donors, P < 0.01; Figure 2C). The GJ blocker appeared to be relatively specific, since preincubation of islets with AGA: (a) reduced the distance between correlated cell pairs from 30 μm to 20 μm, consistent with an effect on GJ-coupling conduction and impedance (38) (Gaussian curve fitted to histogram of n = 3 donors; Figure 2D); (b) did not affect the percentage of GLP-1–responsive cells (Figure 2E); (c) was unable to significantly alter intracellular Ca^{2+} rises in response to 11 mM glucose or 20 mM KCl (Supplemental Figure 3, A and B, respectively); and (d) yielded identical results to experiments in which gene expression was silenced in β cells by greater than 80% using shRNA against human Cx36 (Supplemental Figure 4). GJ blockade severely diminished incretin-stimulated (Figure 2F), but not glucose-stimulated, insulin secretion (117.3 ± 12.7 versus 132.7 ± 18.6 AU, BGA versus AGA, respectively; n = 4 recordings, NS) (Figure 2G).
using the sensitive membrane-tethered zinc (Zn\(^{2+}\)) probe ZIMIR (39), suggesting that GLP-1 may drive insulin release by improving correlated activity within a subpopulation of β cells (Supplemental Videos 2 and 3).

**Lipotoxicity targets the incretin-sensitive β cell subpopulation to impair insulin secretion.** To assess whether this novel mode of incretin action might be targeted by diabetogenic insults, we incubated human islets under lipotoxic conditions (40–42), namely in the presence of high levels of the free fatty acid (FFA) palmitate. The effectiveness of the saturated fatty acid was confirmed by the upregulation of mitochondrial uncoupling protein 2 (UCP2) mRNA expression (1.00 ± 0.04 versus 1.95 ± 0.24 relative mRNA expression, control versus palmitate, respectively; \(P < 0.01\)) (43). As previously observed for rodent islets (44), 72-hour incubation with palmitate markedly reduced Cx36 protein expression in a protein kinase A–dependent (PKA-dependent) manner (24.0 ± 1.6 versus 11.1 ± 1.4% area occupied by Cx36, control versus palmitate, respectively; \(P < 0.05\)) (43). As pre

**Figure 4**

Palmitate disrupts coordinated responses to GIP. (A) β cell responses to stimulation with 10 nM GIP are highly coordinated under control conditions (G11, 11 mM glucose). Top panel: representative Ca\(^{2+}\) traces; red, smoothed; gray, raw. Bottom panel: heatmap depicting minimum (0) to maximum (100) for each cell. (B) As for A, except following 72-hour exposure to palmitate. Note the asynchronous responses in the GIP-responsive population. (C) Lipotoxic conditions significantly disrupt coordinated cell responses to GIP (**\(P < 0.01\), two-way ANOVA; \(n = 11–12\) islets from 3 donors).
secretion by palmitate (Supplemental Figure 6) was not accompanied by decreases in the percentage of active cells (Figure 6A), frequency of Ca$^{2+}$ oscillations (Figure 6, B and C), amplitude and AUC of [Ca$^{2+}$], rises (Figure 6D), or long-term evolutions in coordinated activity (Figure 6E; correlation calculated over 20 to 30 minutes) in response to elevated (11 mM) glucose (n ≥ 4 islets from 3 donors). Likewise, we observed that lipotoxicity did not affect changes in cytosolic free Ca$^{2+}$ detected in response to the non-metabolic depolarizing stimulus tolbutamide (Figure 6F) or the inositol 1,4,5-triphosphate-generating (IP$_3$)-generating muscarinic agonist carbachol (Figure 6H). The coordinated [Ca$^{2+}$], rises elicited by both drugs were not disrupted by FFA exposure (Figure 6G and I; n = 4 recordings) or GJ blockade (Supplemental Figure 11), suggesting that they were likely due to simultaneous depolarization throughout the recorded β cell population.

Incretin-induced upregulation of coordinated cell activity is species specific. To determine whether the above findings in human islets might be recapitulated in a physiological model of in vivo FFA excess, we examined the effects of diet-induced obesity (DIO) in mice. As expected, islets isolated from C57/BL6 mice fed a high-fat diet (HFD) (60% calories from fat) for at least 18 weeks to elevate circulating FFA concentrations presented severe glucose intolerance (Supplemental Figure 12A) and mild downregulation of Cx36 (Supplemental Figure 12B), as previously shown (48), without affecting cell viability (Supplemental Figure 12C; n = 5–10 animals) (49). Over half (53%) of the islets from the HFD animals displayed disorganized Ca$^{2+}$ oscillations in response to high (11 mM) glucose (Supplemental Videos 4 and 5), yet retained apparently normal control GLP-1 responses that resembled those from human islets (Figure 7A). Further analyses of these HFD-responsive islets yielded a significant decrease in the coordination of glucose-induced Ca$^{2+}$ oscillations compared with those from animals fed a normal diet (ND) (90.8 ± 2.9 versus 58.0 ± 5.2% significantly correlated cell pairs before GLP-1 application, ND versus HFD, respectively; n = 8 islets from 4 animals, P < 0.01) (Figure 7B). This deficit was reversed by the application of GLP-1, as evidenced by restoration of normal levels of coordinated cell activity (Figure 7B) and subtly improved ISIS (355.5 ± 27.2 versus 487.4 ± 30.2 AU, ND versus HFD, respectively; n = 12 islets from 4 animals, P < 0.01) (Figure 7C). Calculation of the duty cycle revealed that ISIS in the ND animals was associated with increased time spent in the active phase, rather than any changes in correlated cell-cell Ca$^{2+}$-spiking activity (Figure 7D). These observations could be mimicked by the exogenous application of palmitate to isolated mouse islets (Figure 7, E–G, and Supplemental Figure 13), confirming a direct action of the lipid on the rodent β cell to impair coordinated responses to glucose. Therefore, the exaggerated FFA levels associated with an HFD are unlikely to primarily target ISIS. Instead, the oral glucose intolerance detected in these mice may result from impaired GSIS and/or defective insulin action rather than a diminished incretin effect (49).

**BMI is negatively associated with coordinated responses to GLP-1.** To investigate whether the FFA-impaired incretin responses detected in vitro were present in human islets exposed in vivo to excess circulating lipid, as observed with increasing obesity (50), we examined the relationship between the BMI of individual organ donors and GLP-1–stimulated β cell activity. We detected a significantly negative linear correlation between donor BMI (spanning the normal-obese range) and coordinated ISIS responses to GLP-1 (Figure 8A; R$^2$ = −0.88, P < 0.001). In contrast, the age of the same individuals did not correlate with GLP-1–coordinated β cell responses (Figure 8B; R$^2$ = −0.07, P = 0.37).

**Discussion**

The principal aim of the current study was to determine whether β cell-β cell coordination is an important element in the response of human islets to secretagogues. We show that efficient ISIS from intact human islets relies on driving high levels of coordinated activity in a 3D organized subnetwork of GJ-interlinked β cells. This hitherto unknown mode of incretin action is targeted in vitro by lipotoxicity, an insult strongly associated with T2DM risk and one that has previously been shown to result in defective GSIS (40, 41). Specifically, chronic exposure to elevated FFAs reduces insulin release by disrupting intercellular communications, impeding the timely propagation of signals throughout the GLP-1–sensitive syncytium (see Supplemental Figure 14 for a schematic illustration). Donor BMI was negatively associated with coordinated islet responses to GLP-1, suggesting that this novel
mode of incretin action may also be perturbed by excess circulating lipids in humans.

An increasing body of work indicates that β cell responses to GLP-1 are altered during T2DM in both lean and obese individuals (22–24, 51), changes further compounded by the inheritance of T2DM risk alleles (52). Direct evidence that lipotoxicity represents a primary step in reducing the sensitivity of pancreatic β cells to GLP-1 in human subjects has, however, been difficult to obtain, given the effects of lipotoxicity to raise circulating glucose levels and to decrease GLP-1 secretion from the gut (53). Our results, based on both in vitro and in vivo analyses, strongly suggest that lipotoxicity may impair β cell responses to both GLP-1 and GIP independently of glucotoxicity. The current findings also corroborate recent in vivo studies in which the incretin effect was found to be attenuated in obese individuals with normal glucose tolerance and GLP-1 output (25). Thus, diminished incretin sensitivity to glucose would lead to a decrease in insulin secretion and thus in glucose disposal. As a result, the progressive rise in glucose levels would reduce the circulating levels of incretins (i.e., GLP-1 and GIP), which would, in turn, result in the blunted glucoregulatory effects of GLP-1, thus contributing to the development of impaired glucose tolerance and type 2 diabetes. Accordingly, our observations strongly support the hypothesis that lipotoxicity represents a primary step in reducing the sensitivity of pancreatic β cells to GLP-1 in obese insulin-resistant individuals.
is likely to represent an early event in T2DM pathogenesis in obese individuals, contributing to, rather than being a consequence of, more widespread β cell dysfunction.

The deleterious effects of FFAs on the islet dynamics underlying insulin release appear to be manifested through PKA- and cAMP-dependent inhibition of cell-cell GJ coupling. While our data suggest that lipotoxicity provokes a seemingly paradoxical reduction in cAMP and an elevation in PKA, this should be considered in light of the complexities inherent in this Gs subunit–operated signaling cascade (54). For example, palmitate may increase PKA activity through an AC isoform distinct from those upregulated by forskolin, chronic FFA-induced increases in cAMP/PKA may not reflect the suppression of maximal forskolin-mediated AC stimulation, and lipids can activate PKA via cAMP-independent pathways including those mediated by sphingosine accumulation (55–57). Nevertheless, given that GLP-1 and GIP engage cAMP, a messenger shown to acutely improve electrical transfers between β cells (45), and given that Ca2+ responses to forskolin were blunted by palmitate exposure in our experiments, it is reasonable to hypothesize that FFAs may target the islet dynamics underlying ISIS with two interrelated modes of action: impairment of GJ communications via a chronic effect of PKA on Cx36 protein expression (44), and inhibition of the augmented electrotonic coupling observed in response to fleeting challenge with cAMP-elevating ligands such as GLP-1 (45). Further functional dissection of the exact mechanisms involved will require the use of cAMP-monitoring recombinant probes that can be adapted for cell-specific expression throughout intact human islets (58).

Figure 7
Species specificity of incretin action on coordinated β cell activity. (A) β cells within islets from ND animals display highly coordinated Ca2+ oscillations in response to 11 mM glucose (G11), and synchronicity is not affected by 20 nM GLP-1. Conversely, an HFD disrupts G11-stimulated coordinated β cell activity, but this is restored by the application of incretin. Top panel: representative Ca2+ traces. Bottom panel: heatmap depicting minimum (0) to maximum (100) for each cell. (B) Bar graph demonstrating that the mean percentage of significantly correlated cell pairs is restored to ND values during the application of GLP-1 to islets derived from HFD-fed animals (**P < 0.01 versus before GLP-1 application; Kruskal-Wallis test; n = 8 islets from at least 4 animals). (C) GLP-1–stimulated insulin secretion (G11; 11 mM glucose), as measured using ZIMIR, is subtly improved by an HFD (**P < 0.01 versus ND, Mann-Whitney U test; n = 12 islets from 4 animals). (D) Duty cycle (i.e., time spent in the active phase) is 2-fold higher during the application of GLP-1 to islets from ND animals (**P < 0.01 versus before GLP-1 application; Mann-Whitney U test; n = 4 recordings). (E) As for B, except for the exogenous application of palmitate to islets from animals fed an ND (**P < 0.01 versus before GLP-1 application; Kruskal-Wallis test; n = 8 islets from 6 animals). (F) Representative traces from control- and palmitate-treated islets. (G) As for D, except for the exogenous application of control (BSA plus NaOH) to islets from animals fed an ND (**P < 0.01 versus before GLP-1 application; Mann-Whitney U test).
Although we used chiefly lipotoxic conditions throughout the present study, it remains to be determined whether “glucolipotoxic” conditions (i.e., elevated glucose and fatty acids) (40, 41) exert similar or more severe effects on connectivity. In any case, the mechanisms by which (gluco)lipotoxicity impacts the genes involved in β cell–β cell communication such as Cx36 are still largely obscure, but may involve enhanced peroxisomal fatty acid oxidation, production of ROS, and repression of cAMP-response elements (44, 61).

We asked whether the incretin-regulated changes in β cell cooperativity we observed in human islets might also be present in rodent systems, and thus ultimately be amenable to more straightforward physiological and genetic dissection. β cells within mouse islets are arranged into “small-world” networks and, in response to glucose, display explosive increases in cytosolic Ca^{2+} levels that rhythmically spread throughout the population as autoregenerative oscillatory wavefronts (62–64). Since these activity dynamics underlie insulin secretion, as inferred from animal models presenting with disorganized cell activity (36, 37, 46), attention has been focused on elucidating the mechanisms underlying the intraislet regulation of β cell function in the rodent setting. However, β cells residing within human islets behave in a more stochastic manner, with coordination present only in small cell ensembles and no apparent large-scale synchrony when viewed at the population level (27, 34). Although human islets possess differing paracrine and neural regulation of intercellular communication (29, 30), similarities to their murine counterparts nonetheless exist, such as β cell–specific expression of Cx36 and the extent of β cell dye coupling (35). The current finding that incretin responses in intact human islets display marked GJ dependency therefore raises the intriguing possibility that insulin secretion from human islets is more reliant on an enhancement of coordinated β cell activity than non-sugar secretagogues. Indeed, mice consume small, frequent meals, particularly after lights out, whereas food intake in humans is strongly entrained to the light phase, when it occurs as 3 to 4 meals (65). Thus, an evolutionarily advantageous link between blood glucose levels and food intake may be imprinted on human islets in the form of phylogenetic differences in the mechanisms that regulate incretin responsiveness.

Of note was the observation that obese mice possessed glucose and incretin responses similar to those seen in human islets, since the maintenance of mice on an HFD perturbed coordinated responses to glucose that could be rescued by GLP-1 application. Lipids mildly reduced Cx36 expression in these animals, so the restorative effect of GLP-1 on cell-cell communications may stem from either continued transmission through the remaining GJs, or upregulation of GJ-independent signaling mechanisms (see ref. 66 for a discussion of possible mechanisms). Consequently, rather than representing a paradigm of exposure to excess FFAs, hypertrophied islets from HFD-fed animals may instead more closely model the default human scenario, albeit with subtle differences in the structural and functional regulation of ISIS (26). These species-specific differences may reflect diet-adapted strategies to maintain normoglycemia in the face of nutrient oversupply, since the proportion of energy intake attributable to fat is almost two-fold lower in mice than in humans (~13% [1] versus 25% [7] fat [saturated fat]).

Conversely to our findings in human islets, palmitate has been shown to subtly downregulate receptor mRNA expression in isolated mouse islets (67), although this did not appear to alter the fold change in ISIS. Of interest, an endogenous GLP-1 system has recently been reported in human islets, and tissue isolated from T2DM donors displayed upregulated incretin release (68). Consistent with the latter finding, increased GLP-1R expression may therefore represent a compensatory mechanism to maintain ISIS in the face of compromised GSIS in humans. While these data further underscore the species divergence in incretin signaling, overinterpretation should be avoided in the absence of GLP-1R quantification by Western blotting, an approach currently precluded by the lack of specific antibodies against either mouse or human receptor epitopes (69).

In summary, these results characterize a new aspect of human islet behavior involved in the normal responsiveness to secretagogues and a likely contributor to insulin secretory failure in T2DM. Since reduced GLP-1–stimulated insulin secretion is a feature of T2DM associated with both obesity and risk alleles (22, 25, 52), changes in incretin-regulated β cell cooperativity, which we describe here, may provide a unifying mechanism through which multiple factors such as lifestyle and genes can impact insulin release to reduce β cell function in humans.

Methods

Animal studies. Animals were maintained in a pathogen-free facility under a 12-hour light/12-hour dark cycle with free access to water and food. For the HFD treatment, 8-week-old C57BL/6 mice were placed on chow containing 60% wt/wt fat content (Lillico Biotechnology). At 15 weeks of feeding, the animals were starved overnight before being subjected to an intraperitoneal glucose tolerance test (IPGTT) (1 g/kg D-glucose) to confirm the efficacy of the HFD in promoting glucose intolerance.

Cell culture. Human islets obtained from normoglycemic donors at isolation centers in Oxford, Geneva, Milan, and Pisa were cultured in RPMI supplemented with 5.5 mM D-glucose, 10% FCS, 100 U penicillin, 100 μg streptomycin, and 0.25 μg/mL fungizone (37°C, 5% CO2). Islets were treated for 72 hours with either control (BSA plus NaOH), 0.5 mM BSA-conju-
 gated palmitate or 0.5 mM BSA-conjugated palmitate plus 10 μM H89 (PKA inhibitor; Tocris). For the GJ inhibition studies, islets were pre-incubated for 1 hour with AGA or its inactive analog BGA (both 20 μM; Sigma-Aldrich) before imaging. Studies have shown this dose to effectively disrupt GJ signaling in intact islets (70). Mice were euthanized by cervical dislocation, and pancreatic islets were isolated by collagenase digestion (71). Islets were cultured for 24 to 48 hours as above, but in RPMI supplemented with 11 mM instead of 5.5 mM glucose, and containing no fungizone.

Calcium imaging. Isolated islets were incubated (37°C, 95% O2/5% CO2) for 30 minutes in fluo-2-AM (10 μM) diluted with a mixture of DMSO (0.01%, wt/vol), and procloric acid (0.001%, wt/vol; all from Invitrogen) in a bicarbonate buffer solution (120 mM NaCl, 4.8 mM KCl, 1.25 mM NaH2PO4, 24 mM NaHCO3, 2.5 mM CaCl2, 1.2 mM MgCl2, and 3 mM D-glucose). Following acclimatization to the indicated glucose concentration, islets were placed on glass coverslips in a custom-manufactured aluminum chamber (Digital Pixel) mounted on a Zeiss Axiovert coupled to a Nipkow spinning-disk head (Yokogawa CSU-10) and equipped with objectives adjusted for chromatic aberration (10×–20×/0.3–0.5 NA, EC Plan-Neofluar; Zeiss). Whole-islet multicellular Ca2+ imaging was performed as previously described (31). Briefly, Ca2+ dynamics were captured with cellular resolution from the 50–200 fluo-2–loaded cells residing within the first and second layers of the intact islets. Use of a spinning disk to rapidly scan large areas of the islet allowed events to be recorded for long periods with minimal phototoxicity. A solid-state laser (CrystaLaser) controlled by a laser-merge module (Spectral Applied Physics) provided wavelengths of 491 nm to excite fluo-2 (rate = 0.5Hz; exposure time = 263 ms). Emitted light was filtered at 525/50 nm, and images were captured by a highly sensitive 16-bit, 512 × 512 pixel back-illuminated EM-CCD camera (ImageEM 9100-13; Hamamatsu). Velocity software (PerkinElmer) provided the user interface. During recording, islets were maintained at 35°C to 36°C and continuously irrigated with bicarbonate buffer aerated with 95% O2/5% CO2-Glut 1 (7-36 human amide fragment), tolbutamine, forskolin, carbachol, AGA, BGA (all from Sigma-Aldrich), GIP (human), and exendin 9-39 (both from Bachem) were delivered via the perfusion system at the doses indicated in the figures and accompanying legends.

Real-time imaging of insulin release. Insulin release was monitored using ZIMIR as previously described (39). Briefly, before imaging, islets were incubated for 2 hours in ZIMIR (1 mM), a membrane-bound probe that fluoresces upon binding of zinc (Zn2+) coreleased with insulin from granules. During recording, islets were irrigated with bicarbonate buffer containing 1 μM EGTA to improve the signal-to-noise ratio (SNR) without affecting intracellular Ca2+ or Zn2+ concentrations (39). Excitation was delivered at 491 nm (rate = 0.2 Hz; exposure = 1 second), and emitted signals were collected at 525/50 nm. Islets were arbitrarily subdivided into 10 to 20 regions, and the normalized (F/F0) intensity over time was calculated for each region. Islet-wide insulin release dynamics were then represented by the mean intensity over time and the AUC, calculated by ignoring peaks less than 20% of the distance from minimum-to-maximum y to account for fluctuations in the baseline due to the SNR.

Insulin secretion assay. Six human islets were incubated in duplicate for 30 minutes in 1 mL of Krebs-HEPES-bicarbonate (KH) buffer solution (130 mM NaCl, 3.6 mM KCl, 1.5 mM CaCl2, 0.5 mM MgSO4, 0.5 mM NaH2PO4, 2 mM NaHCO3, 10 mM HEPES, and 0.1% [wt/vol] BSA, pH 7.4) at 37°C containing the indicated glucose and GLP-1 concentrations. A specific RIA for human insulin (Millipore) was used to measure hormone released into the medium. Following 2 hours of pentobarbital, and immediately perfused via the left ventricle with 4% PFA. The pancreas was then cryosectioned and immunolabeled for insulin (guinea pig anti-insulin IgG; 1:200; Ventrex), glucagon (mouse anti-glucagon IgG; 1:2,000; Sigma-Aldrich), and FITC to enhance the exendin signal (rabbit anti-FITC IgG; 1:100; Invitrogen). The secondary antibodies used were anti-guinea pig IgG coupled to Dylight 405 (1:800; Jackson ImmunoResearch Laboratories), anti-mouse IgG coupled to TRITC (1:500; SouthernBiotech), and anti-rabbit IgG coupled to Alexa 488 (1:500; Invitrogen) for insulin, glucagon, and exendin, respectively. To estimate the GLP-1R number at the cell surface in human islets, 100 nM exendin-4 FITC diluted in PBS was applied for 1 hour at 4°C (to minimize internalization) before rinsing and processing for immunohistochemistry. To assess total GLP-1R binding, islets were permeabilized with 1.5 mM Triton X-100 before application of exendin-4 FITC. Specificity was verified by preincubating islets for 10 minutes with excess 1 μM GLP-1.

Lentiviral delivery of shRNA. Commercially validated lentiviral particles carrying shRNA expression constructs against human GJD2 were acquired from Sigma-Aldrich (NM_020660; clone IDs: TRCN000074089, TRCN000074092, TRCN0000413941, and TRCN000436198). MOI was calculated using Turbo-GFP particles (Sigma-Aldrich). Specificity of GJD2 gene silencing was confirmed using quantitative PCR (qPCR). In all cases, nontargeting lentiviral particles containing scrambled shRNA were used as negative controls (Sigma-Aldrich).

Real-time PCR. Total RNA was extracted in TRIzol reagent and reverse transcribed using a high-capacity cDNA reverse transcription kit (both from Invitrogen). The relative mRNA abundance was quantified by qPCR using SYBR Green Master Mix and a 7500 Fast real-time PCR engine (both from Invitrogen). Primers (Supplemental Table 2) were designed not to span genomic DNA sequences using Primer3. Specificity was validated using a dissociation curve, and linear amplification of both target and housekeeping genes was tested using a dilution series. The expression of each gene was normalized to cyclophilin A (Ppia), and relative changes in mRNA expression versus control were calculated using the comparative Ct method (2–ΔΔCt).
Western immunoblotting. Islets were washed in ice-cold PBS before lysis in precipitation assay buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) and sonication. Total protein extracts were resolved using a 12% SDS-PAGE gel (12% Tris-acrylamide), wet transferred onto a PVDF membrane, and subjected to immunoblotting using either rabbit anti-Cx36 (1:50; Invitrogen) or mouse monoclonal antitubulin (1:5,000; Sigma-Aldrich clone B-5-1-2). Revelation was performed using HRP-linked anti-rabbit antibodies (1:10,000; GE Healthcare) and a proprietary ECL detection reagent.

Live/dead assay. Islets were incubated for 15 minutes in PBS containing 3 μM calcein AM (Invitrogen) and 2.5 μM propidium iodide (PI) (Sigma-Aldrich) before detection of absorbance/emission at 491/525 nm and 561/620 nm, respectively. The islet area occupied by dead cells (PI) was calculated and expressed as a unitary ratio versus that occupied by live cells (calcein).

Correlation analysis. Correlation analyses were performed using a Pearson R coefficient as previously detailed (72). Briefly, individual fluo-2–loaded cells were identified using a region of interest (ROI), and intensity over time traces and Cartesian (x,y) coordinates were extracted. The resulting datasets were imported into R (R project, R Development Core Team) and IgorPro (Wavemetrics), baseline trends were removed by fitting a linear equation, and intensity values were normalized. GLP-1–responsive cells were manually filtered on the basis of a sustained rise in [Ca2+]; levels above a 10% threshold to account for SNR. Subsequent to this, the one-sided correlation function between all possible cell pair combinations (excluding the auto-correlation) was assessed using the Pearson’s product moment correlation defined by Equation 1, where x and y represent cells 1 and 2, respectively.

\[
r = \frac{\sum xy}{\sqrt{\sum x^2 \sum y^2}}
\]

(Equation 1)

Significance (P < 0.05) was determined against the expected t distribution of independent R values (with two degrees of freedom). The location of significantly correlated cell pairs was displayed on a weighted graph constructed using the Cartesian (x,y) coordinates of each cell. Phase maps were compiled by converting the normalized intensity (minimum = 0; maximum = 100) of each cell to a color corresponding to a user-generated light-dark 256-color ramp.

Frequency and duty cycle analysis. Ca2+-spiking frequency was measured as previously described (31). Briefly, power spectrums based on the fast Fourier transform (FFT) were constructed for each cell, a confidence interval of 95% was imposed, and the frequency with the highest significant power was extracted. A histogram of frequency (mHz) was then plotted for each cell to a color corresponding to a user-generated light-dark 256-color ramp. The location of significantly correlated cell pairs was displayed on a weighted graph constructed using the Cartesian (x,y) coordinates of each cell. Phase maps were compiled by converting the normalized intensity (minimum = 0; maximum = 100) of each cell to a color corresponding to a user-generated light-dark 256-color ramp.

\[
D = \frac{\tau}{T}
\]

(Equation 2)

Statistics. In all cases, data distribution was determined using a D’Agostino omnibus test. Non-Gaussian data were analyzed using either a Mann-Whitney U test or, where multiple comparisons were required, a Kruskal-Wallis test (followed by a Dunn’s post-hoc test). Normally distributed data were analyzed using a two-tailed Student’s t test or one-way ANOVA followed by a Bonferroni’s post-hoc test (accounting for degrees of the freedom). Between-treatment effects were assessed using two-way ANOVA followed by a Bonferroni’s post-hoc test. Linear correlations were calculated using regression analyses. Analysis was performed using R, GraphPad Prism (GraphPad Software), IgorPro, and MATLAB (MathWorks). P < 0.05 was considered statistically significant, and values represent the mean ± SEM unless otherwise stated.

Study approval. All studies involving human tissue were approved by the National Research Ethics Committee (NRES) London (Fullham) “Signal transduction in isolated human islets: regulation by glucose and other stimuli” (REC 07/H0711/114). Islets were isolated under the approval of NRES Oxfordshire (REC 09/H0605/2) (Oxford, United Kingdom), Comitato di Bioetica Azienda Ospedaliero-Universitaria Pisana (34058) (Pisa, Italy), Comitato Etico Istituto Scientifico San Raffaele (Milan, Italy), and the Central Institutional Review Board on Clinical Research of Geneva University Hospitals (CER 05-028 [05-065]) (Geneva, Switzerland). Where required, consent from next of kin was obtained before use of tissue for scientific research. Animal procedures were approved by the Home Office according to the Animals (Scientific Procedures) Act 1986 of the United Kingdom (PPL 70/7349) and/or the Geneva Veterinary Office (Geneva, Switzerland; authorization 1034/3552/1).

Acknowledgments

Studies were supported by a Diabetes UK R.D. Lawrence Research Fellowship (12/0004431; to D.J. Hodson); a Wellcome Trust Senior Investigator grant (WT098424AA/A); the MRC Programme (MR/J0003042/1); a Diabetes UK Project Grant (11/0004210); and Royal Society Wolfson Research Merit Awards (to G.A. Rutter); the Swiss National Science Foundation (310030_141162; and CR3213_129987); Juvenile Diabetes Research Foundation (JDRF) (5-2012-281; 99-2012-775) and EU FP7 (BETAIMAGE 222980; BETATRAIN 289932) awards (to P. Meda); and JDRF (37-2011-21) and NIH (R01-GM077593) awards (to W-H. Li). The work leading to this publication has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement 155005 (IMI-Med), resources of which are composed of financial contributions from the European Union’s Seventh Framework Programme (FP7/2007-2013) and EFPIA companies’ kind contribution (to G.A. Rutter and P. Meda). Isolation of human islets was supported by JDRF (JRDF) (5-2012-281; 99-2012-775) and EU FP7 (BETAIMAGE 222980; BETATRAIN 289932) awards (to P. Meda); and JDRF (37-2011-21) and NIH (R01-GM077593) awards (to W-H. Li). The work leading to this publication has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement 155005 (IMI-Med), resources of which are composed of financial contributions from the European Union’s Seventh Framework Programme (FP7/2007-2013) and EFPIA companies’ kind contribution (to G.A. Rutter and P. Meda). Isolation of human islets was supported by JDRF awards (31-2008-416, to D. Bosco and 31-2008-413 to L. Piemonti) (European Consortium for Islet Transplantation, ECIT). We are also grateful to Francesca Semplici, Timothy Pullen, and Gabriela Da Silva Xavier for their useful advice on molecular biology. Last, we thank Patrice Mollard, Pierre Fontanaud, and Francois Molino of the Institut de Genomique Fonctionnelle, Montpellier, France, for their assistance with analysis.

Received for publication December 21, 2012, and accepted in revised form July 11, 2013.

Address correspondence to: David J. Hodson, Section Cell Biology, Department of Medicine, Imperial College London, London SW7 2AZ, United Kingdom. Phone: 44.0.20.7594.1713; Fax: 44.0.20.7594.3351; E-mail: d.hodson@imperial.ac.uk. Or to: Guy A. Rutter, Section of Cell Biology, Department of Medicine, Imperial College London, London SW7 2AZ, United Kingdom. Phone: 44.0.20.7594.3340; Fax: 44.0.20.7594.3351; E-mail: g.rutter@imperial.ac.uk.


