Role of \( K_{\text{ATP}} \) Channels in Glucose-Regulated Glucagon Secretion and Impaired Counterregulation in Type 2 Diabetes

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SUMMARY

Glucagon, secreted by pancreatic islet \( \alpha \) cells, is the principal hyperglycemic hormone. In diabetes, glucagon secretion is not suppressed at high glucose, exacerbating the consequences of insufficient insulin secretion, and is inadequate at low glucose, potentially leading to fatal hypoglycemia. The causal mechanisms remain unknown. Here we show that \( \alpha \) cell \( K_{\text{ATP}} \)-channel activity is very low under hypoglycemic conditions and that hyperglycemia, via elevated intracellular ATP/ADP, leads to complete inhibition. This produces membrane depolarization and voltage-dependent inactivation of the Na\(^+\) channels involved in action potential firing that, via reduced action potential height and Ca\(^{2+}\) entry, suppresses glucagon secretion. Maneuvers that increase \( K_{\text{ATP}} \) channel activity, such as metabolic inhibition, mimic the glucagon secretory defects associated with diabetes. Low concentrations of the \( K_{\text{ATP}} \) channel blocker tolbutamide partially restore glucose-regulated glucagon secretion in islets from type 2 diabetic organ donors. These data suggest that impaired metabolic control of the \( K_{\text{ATP}} \) channels underlies the defective glucose regulation of glucagon secretion in type 2 diabetes.

INTRODUCTION

Glucagon and insulin are the body’s principal plasma glucose-regulating hormones. They are secreted from the \( \alpha \) and \( \beta \) cells of the pancreatic islets, respectively. Physiologically, glucagon is released in response to a fall in plasma glucose levels, an increase in amino acids, and \( \beta \)-adrenergic stimulation (Gromada et al., 2007). Diabetes is a bihormonal disorder involving both inadequate insulin secretion and defective glucagon secretion. The glucagon secretory defects include oversecretion at high glucose (when it is not needed) and inadequate release at low glucose (when it is needed) (Cryer, 2002; Unger and Cherrington, 2012).

Whereas the cellular regulation of insulin secretion is fairly well understood (Remedi and Nichols, 2009; Seino et al., 2011), much less is known about the control of glucagon secretion (Gaisano et al., 2012). Hypotheses for the regulation of glucagon secretion include paracrine effects, mediated by factors released from neighboring insulin-secreting \( \beta \) cells or somatostatin-secreting \( \delta \) cells, or innervation (Gromada et al., 2007). However, in both human and rodent islets, glucagon secretion is strongly inhibited by glucose concentrations that have little stimulatory effect on insulin secretion (Walker et al., 2011), and glucose remains capable of suppressing secretion following pharmacological or immunological inhibition of somatostatin signaling (de Heer et al., 2008; Vieira et al., 2007). Moreover, glucagon secretion responds normally to hypoglycemia after denervation of the pancreas (Sherck et al., 2001). These considerations suggest that \( \alpha \) cells, in addition to being under paracrine control, possess an intrinsic glucose-sensing mechanism. This remains poorly defined, but studies on \( K_{\text{ATP}} \)-channel knockout mice indicate that \( K_{\text{ATP}} \)-channels are somehow involved (Cheng-Xue et al., 2013; Gromada et al., 2004; Muñoz et al., 2005; Shiota et al., 2005) and the inhibitory effect of high glucose can be reversed by low concentrations of the \( K_{\text{ATP}} \)-channel activator diazoxide (Göpel et al., 2000b; MacDonald et al., 2007). In pancreatic \( \beta \) cells, closure of these channels by metabolically generated ATP leads to membrane depolarization, electrical activity, and insulin secretion. It is not immediately evident, however, how regulation of the same channels by glucose in \( \alpha \) cells could suppress glucagon secretion. To date, most studies have failed to detect an effect of glucose on \( \alpha \) cell \( K_{\text{ATP}} \)-channel activity (Barg et al., 2000; Bokvist et al., 1999; Quoix et al., 2009; Ramracheya et al., 2010), but one study reported a small glucose-induced decrease in \( K_{\text{ATP}} \) channel activity that, paradoxically, was associated with stimulation rather than inhibition of glucagon secretion (Olsen et al., 2005). Notably, all these
K<sub>ATP</sub> Channels and Glucagon Secretion

**RESULTS**

Glucose Regulates Glucagon Secretion by an Intrinsic, Nonparacrine Mechanism

We first established that the inhibitory effect of glucose on glucagon secretion is secondary to glucose metabolism. Mannohexotulose, an inhibitor of glucose phosphorylation, abolished the inhibitory effect of 6 mM glucose on glucagon secretion, without affecting hormone release at 1 mM glucose (Figure 1A).

We used the fluorescent probe Perceval to measure the cytosolic ATP/ADP ratio [ATP]/[ADP]<sub>cyt</sub> in α cells of intact mouse islets (Figure 1B). Addition of the mitochondrial uncoupler FCCP promptly decreased [ATP]/[ADP]<sub>cyt</sub>. On average, glucose increased [ATP]/[ADP]<sub>cyt</sub> (measured as relative increase in Perceval fluorescence above basal) by 33% ± 2% (n = 44; p < 0.02, Mann-Whitney U test for independent samples). In these experiments, α cells were identified by tdRFP fluorescence. We have considered the possibility that tdRFP might interfere with the ATP measurements. However, we do not think this is the case. First, background tdRFP fluorescence was stable and unaffected by glucose or FCCP (Figure 1B). Second, effects of glucose in α cells identified by spontaneous oscillations in [Ca<sup>2+</sup>]<sub>i</sub> at 1 mM glucose were very similar to those seen in α cells identified by tdRFP fluorescence. Thus, we conclude that glucose increases ATP in α cells. These findings are in agreement with earlier reports of a small (10%–20%) increase in cytoplasmic [ATP] (Ishihara et al., 2003; Ravier and Rutter, 2005) and of total ATP and ADP (Detimary et al., 1998) in response to glucose in α cells using alternative methods of measurement.

Both somatostatin and insulin have been proposed to mediate the inhibitory effects of glucose on glucagon secretion in intact islets (Unger and Cherrington, 2012; Unger and Orci, 2010). The somatostatin receptor subtype 2 (SSTR2) antagonist CYN154806 increased glucagon secretion at both 1 and 6 mM glucose, suggesting α cells are under tonic somatostatin inhibition. In freshly isolated islets, tolbutamide (unlike what was previously observed in cultured islets; Cheng-Xue et al., 2013) remained inhibitory on glucagon secretion when somatostatin signaling was acutely inhibited using CYN154806 (Figure 1C).

Figure 1D shows the reciprocal increase in insulin secretion and decrease in glucagon secretion produced when glucose is lowered from 6 mM to 1 mM. Glucagon secretion was maximally stimulated before any detectable inhibition of insulin secretion. Tolbutamide produced a transient 10-fold stimulation of insulin secretion and mimicked the inhibitory effect of glucose on
glucagon secretion, but glucagon secretion remained suppressed even when stimulated insulin release had decayed by 80%. The report that glucagon secretion evoked by insulin-induced hypoglycemia is not very different in control mice and in mice lacking insulin receptors in α cells (Kawamori et al., 2009) provides additional evidence for a dissociation between insulin and glucagon secretion.

Collectively, these data suggest that glucose inhibits glucagon secretion by mechanisms that do not require paracrine effects of insulin or somatostatin (or signals coreleased with these hormones). However, paracrine mechanisms may become functionally more significant under physiological situations associated with strong stimulation of insulin and somatostatin secretion.

Effects of Glucose and Pharmacological Modulators of K<sub>ATP</sub> Channel Activity on α Cell Electrical Activity

Unlike insulin-secreting β cells and somatostatin-secreting δ cells (Göpel et al., 2000a), pancreatic α cells in intact mouse islets exposed to 1 mM glucose (i.e., when glucagon secretion is stimulated) generate spontaneous action potentials (Figure 2A). The frequency, the peak voltage, and the most negative membrane potential attained during action potential firing averaged 1.4 ± 0.3 Hz, 2 ± 3 mV, and −54 ± 2 mV (n = 9). Corresponding values in the presence of 6 mM glucose (when glucagon secretion is maximally suppressed) were 3.0 ± 0.5 Hz (p < 0.02), −7 ± 2 mV (p < 0.002), and −45 ± 2 mV (p < 0.01; n = 9). Averaged action potentials (see Experimental Procedures) recorded under the indicated experimental conditions are shown on the right on an expanded time base.

Tolbutamide mimicked the effect of elevating glucose (Figure 2B). It depolarized α cells by 11 ± 3 mV (p < 0.02), reduced action potential peak voltage by 17 ± 6 mV (p < 0.02), and increased firing frequency to 3.3 ± 0.7 Hz (p < 0.02; n = 5).

The effect of the K<sub>ATP</sub>-channel activator diazoxide on α cells exposed to 6 mM glucose was concentration dependent (Figure 2C). At 100 μM, it hyperpolarized α cells to −74 ± 4 mV (p < 0.01; n = 4) and abolished action potential firing. At 1 μM it hyperpolarized α cells by 9 ± 2 mV (p < 0.01) yet increased action potential peak voltage by 7 ± 2 mV (p < 0.02, n = 5).

The maximum rate of depolarization (dV/dt) during the upstroke of the action potential averaged 37 ± 6 V/s (n = 11) at 1 mM glucose, 15 ± 3 V/s (n = 11; p < 0.001 versus 1 mM glucose) at 6 mM glucose (or 0.2 mM tolbutamide), and 33 ± V/s (n = 5; p < 0.02 versus 6 mM glucose) in the presence of 6 mM glucose and 1 μM diazoxide. Corresponding values for the maximum rate of repolarization (dV/dt<sub>r</sub>) averaged −24 ± 3 V/s, −13 ± 1 V/s (p < 0.01 versus 1 mM glucose), and −31 ± 6 V/s (p < 0.05 versus 6 mM glucose). We attribute the slower repolarization in the presence of glucose or tolbutamide to reduced voltage-dependent activation of the K<sup>+</sup>-channels involved in action potential repolarization when spike height is reduced.

In a small number of experiments (4 of 29 cells), elevating glucose transiently hyperpolarized the α cell and suppressed action potential firing (Figure S1A available online). This could be antagonized by CYN154806, suggesting it may result from somatostatin released by neighboring δ cells (Figure S1B).

Some α cells hyperpolarized spontaneously in the continued presence of 1 mM glucose (Figure S1C). This repolarization and suppression of electrical activity lasted ~5 min, after which the α cell depolarized and action potential firing resumed. Spontaneous membrane potential oscillations were seen in α cells exposed to 1 mM glucose in 60% of experiments. These probably account for the [Ca<sup>2+</sup>] oscillations with a period of ~5 min seen in isolated islets at low glucose (Figure S1D). The frequency and amplitude of these oscillations were little affected by 6 mM glucose, as previously reported (Le Marchand and Piston, 2010).
The reported >5-fold greater ATP sensitivity of K\textsubscript{ATP}-channel activity in \( \alpha \) cells than in \( \beta \) cells (Leung et al., 2005) might contribute to the strong tonic inhibition of K\textsubscript{ATP}-channels in \( \alpha \) cells.

**Relationship between K\textsubscript{ATP} Channel Activity and Glucagon Secretion**

Diazoxide activated K\textsubscript{ATP}-channels inhibited by glucose, with half-maximal stimulation occurring at 29 ± 6 \( \mu \)M (n = 5; Figure 3B). Glucagon secretion was measured in the presence of 6 mM glucose (to maximally inhibit secretion) and increasing concentrations of diazoxide. This enables the relationship between K\textsubscript{ATP} conductance (\( G \), determined as above) and glucagon secretion to be determined over a wider range of conductance than can be obtained by varying glucose concentration (Figure 3C). The G-secretion relationship was bell shaped, being maximal at \( \sim 280 \) pS (3 \( \mu \)M diazoxide), where both \( G \) and secretion were similar to that at 1 mM glucose. Importantly, both small (\( \sim 100 \) pS) increases and decreases in \( G \) inhibited glucagon secretion. The ability of diazoxide to reverse the effects of glucose cannot be explained by relief from paracrine suppression of glucagon secretion by insulin or somatostatin, as glucose-induced release of these hormones is unaffected by diazoxide concentrations as high as 30 \( \mu \)M and 200 \( \mu \)M, respectively (MacDonald et al., 2007; Zhang et al., 2007).

The data of Figure 3C confirm our earlier finding (MacDonald et al., 2007), that low concentrations of diazoxide antagonize the inhibitory effect of glucose on glucagon secretion. We also tested the effects of glucose and diazoxide in the presence of two different amino acid mixtures, AAM\( a \) (2 mM) and AAM\( b \) (6 mM), designed to simulate the conditions occurring during fasting (Rudman et al., 1989) and following a protein-rich meal, respectively (Figure 3D). AAM\( a \) did not detectably enhance glucagon secretion beyond that evoked by 1 mM glucose. Increasing glucose to 6 mM in the presence of AAM\( a \) inhibited glucagon secretion as strongly as it did under control conditions, an effect that was prevented by diazoxide. In fact, diazoxide stimulated glucagon secretion in the presence of 6 mM glucose. AAM\( b \) stimulated glucagon secretion \( \sim 4 \)-fold at 1 mM glucose and elevation of glucose to 6 mM reduced glucagon secretion by >60%. Again, no inhibitory effect of glucose was observed in the presence of 3 \( \mu \)M diazoxide. These observations argue that K\textsubscript{ATP}-channel closure plays a key role in the modulation of glucagon secretion by glucose in both the absence and presence of amino acids.

**Membrane Potential-Dependent Reduction of Action Potential Height**

Figures 2 and 3 suggest K\textsubscript{ATP}-channel closure leads to membrane depolarization and reduced action potential amplitude.
We investigated the relationship between interspike membrane potential and the voltage at the peak of the action potential (Figures 4A and 4B). Peak voltage decreased with interspike depolarization, from +18 ± 6 mV at negative membrane potentials (below −60 mV) to −14 ± 2 mV at depolarized potentials (above −40 mV), being half-maximal at −52 ± 3 mV with a slope factor of 4 ± 1 mV/n (n = 4 cells from four different islets and three different mice; Figure 4C). Using these values, we estimate that the 9 mV depolarization produced by increasing glucose from 1 mM to 6 mM will reduce action potential peak voltage from +60 mV to +52 mV (n = 12 action potentials for each data point). The line is a Boltzmann fit to the mean data with a midpoint of −52 mV. The red arrow indicates the decrease in peak voltage predicted from the 9 mV glucose-induced depolarization.

Action potential firing in α cells depends on the opening of voltage-gated Na⁺-channels (Göpel et al., 2000b). We reasoned that glucose-induced membrane depolarization might reduce action potential height by voltage-dependent inactivation of Na⁺-channels. In agreement with this idea, the Na⁺-channel blocker TTX (0.1 μg/ml) reduced action potential peak voltage in α cells exposed to 1 mM glucose by 14 ± 4 mV (n = 5; p < 0.01; Figure 4E), reduced dV/dt from 37 ± 12 V/s to 11 ± 2 V/s (p < 0.05 versus no TTX), and reduced dV/dt from −22 ± 4 V/s to −13 ± 3 V/s (p < 0.05 versus no TTX). It also inhibited glucagon secretion at 1 mM glucose by ~40% (Figure 4F). The peak Na⁺-current amplitude in identified α cells decreased steeply at potentials positive to −60 mV (Figure 4F); the relationship between membrane potential and peak Na⁺-current predicts that the 9 mV glucose-induced depolarization would reduce the fraction of active Na⁺-channels (hₐ) from 0.7 to 0.3 (red arrow). These data argue that glucose-induced membrane depolarization mediates its effect on action potential height via (partial) voltage-dependent inactivation of voltage-gated Na⁺-channels.
Glucose Inhibits Glucagon Secretion by Reducing P/Q-Type Ca\textsuperscript{2+} Channel Activation

Figure 5A summarizes the voltage dependence of exocytosis in \( \alpha \) cells in intact islets. The relationship predicts that the 9 mV reduction in spike height produced by glucose reduces exocytosis by 75\%. Glucagon exocytosis is dependent on Ca\textsuperscript{2+} entry via voltage-gated Ca\textsuperscript{2+}-channels (see Figure S3). The P/Q type Ca\textsuperscript{2+}-channel blocker \( \alpha \)-agatoxin mimicked the inhibitory effect of 6 mM glucose (Figure 5B) and inhibited depolarization-evoked exocytosis (Figure 5C). The reduction in action potential height produced by 6 mM glucose resulted in 66\% ± 10\% (n = 5; \( p < 0.01 \)) inhibition of the peak P/Q type Ca\textsuperscript{2+}-current evoked by simulated action potentials (Figure 5D). During brief depolarizations (≤50 ms), exocytosis proceeded only during the depolarization (Figure 5E), and not subsequent to it (cf. Zhang et al., 2007). This suggests exocytosis in \( \alpha \) cells is controlled by rapid, local [Ca\textsuperscript{2+}]\textsubscript{i} increases, close to the Ca\textsuperscript{2+}-channel, and thus echoes Ca\textsuperscript{2+}-channel activity. Accordingly, exocytosis elicited by short depolarizations was hardly affected by inclusion of the Ca\textsuperscript{2+}-chelator EGTA (1 mM) in the intracellular medium (Figure 5F). On average, 50 ms depolarizations evoked capacitance increases of 14 ± 6 fF (n = 8) and 11 ± 2 fF (n = 5) in the presence of 50 \( \mu \)M and 1 mM intracellular EGTA, respectively. However, exocytosis evoked by 300 ms depolarizations was reduced from 80 ± 20 fF to 30 ± 11 fF (\( p < 0.05 \)), suggesting that Ca\textsuperscript{2+} diffusion during longer depolarizations may trigger exocytosis of granules not situated in the immediate vicinity of the Ca\textsuperscript{2+}-channels.

Glucose (6 mM) did not inhibit \( \alpha \) cell exocytosis evoked by membrane depolarization to 0 mV in \( \alpha \) cells with/without 200 \( \mu \)M \( \alpha \)-agatoxin (\( n = 4 \)). **\( p < 0.05 \) versus no \( \alpha \)-agatoxin.

Glucose (6 mM) did not inhibit \( \alpha \) cell exocytosis evoked by membrane depolarization to 0 mV in \( \alpha \) cells with/without 200 \( \mu \)M \( \alpha \)-agatoxin (\( n = 4 \)). **\( p < 0.05 \) versus no \( \alpha \)-agatoxin.

Effects of K\textsubscript{ATP} Channel Mutations Associated with Reduced ATP Sensitivity on Glucagon Secretion

An increase in K\textsubscript{ATP}-channel activity either stimulates or inhibits glucagon secretion, depending on the magnitude of the increase in the K\textsubscript{ATP} current (Figure 3C). The question then arises as to whether gain-of-function mutations found in patients with neonatal diabetes (Gloyn et al., 2004) affect glucagon secretion. We addressed this by generating mice expressing K\textsubscript{ATP}-channels with reduced ATP-sensitivity in \( \alpha \) cells identified by expression of tdRFP. These had normal body weight (data not shown) and fasting blood glucose levels close to wild-type levels; however, glucagon secretion was reduced by >50% in mutant \( \alpha \)-V59M mice. These mice exhibited mild glucose intolerance (Figures S4A and S4B) and increased insulin sensitivity (Figure S4C).

In 1 mM glucose, the membrane conductance (G) was >13-fold larger in metabolically intact \( \alpha \) cells in islets isolated from \( \alpha \)-V59M mice than in control islets (\( r = 1.5 \) nS/pF versus 0.11 nS/pF; \( p < 0.03 \); \( \alpha \) cells identified by expression of tdRFP). Whereas 6 mM glucose reduced G in control \( \alpha \) cells by 17% ± 5%, no reduction was detected in \( \alpha \)-V59M \( \alpha \) cells (Figure 6A). Tolbutamide reduced G by >50% in mutant \( \alpha \) cells (Figure 6B), but it still remained >4-fold higher than in control \( \alpha \) cells.

We next measured \( \alpha \) cell electrical activity in intact control and \( \alpha \)-V59M islets. In contrast to control cells (which resemble wild-type cells), only 20% (\( n = 10 \)) of \( \alpha \)-V59M \( \alpha \) cells were electrically
active in 1 mM glucose. The other 80% were inactive and hyperpolarized (−79 ± 3 mV). When glucose was elevated to 15 mM, two cells depolarized and generated electrical activity, five were refractory to glucose but responded to tolbutamide, and one did not respond to either agent (Figure 6C).

Glucagon content was similar in wild-type and α-V59M islets, averaging 1,589 ± 213 pg/islet and 2,093 ± 207 pg/islet, respectively. Unexpectedly, although 80% of α-V59M α cells were electrically silent at 1 mM glucose, glucagon secretion from α-V59M islets was not less than from control islets (Figure 6D). Notably, the inhibitory effect of glucose was halved in α-V59M islets (−18% versus −35%) whereas tolbutamide was as inhibitory as in control islets (−30%). In control islets, adrenaline (5 μM) stimulated glucagon secretion ~4-fold, but this effect was strongly reduced in α-V59M islets (Figure 6E); the adrenaline-induced stimulation averaged 7 ± 1 and 24 ± 4 pg/islet/hr (p < 0.05), respectively. By contrast, glucagon secretion evoked by 70 mM K+ was 2.9-fold larger in α-V59M than in wild-type islets (Figure 6F).

A Small Increase in K_ATP Channel Activity Mimics the Effects of Type 2 Diabetes on Glucagon Secretion

Type 2 diabetes (T2D) is associated with loss of glucose-induced suppression of glucagon secretion--indeed, stimulation may occur instead (Dunning et al., 2005). We evaluated if this disturbance is intrinsic to the islet, using islets isolated from T2D and non-diabetic (ND) organ donors (Figure 7A). In ND islets, glucose (6 mM) inhibited glucagon secretion by ~50%. No inhibition by glucose was seen in T2D islets. In fact, glucose stimulated glucagon secretion in 5 of the 10 T2D preparations tested but in only 4 of 46 ND preparations (p < 0.001 by χ²). Glucagon content was 2.5-fold higher in T2D islets than in ND islets (2,457 ± 461 pg/islet versus 985 ± 105 pg/islet; p < 0.01). The relative effect of glucose on glucagon secretion in ND and T2D islets is summarized in Figure 7F. On average, glucose inhibited glucagon secretion by 43% ± 6% in ND islets but tended to stimulate glucagon secretion by 15% ± 24% in T2D islets. In the five preparations where glucose had the least inhibitory effect, glucose enhanced glucagon secretion by 74% ± 28%. In the remaining five preparations, glucose inhibited glucagon secretion by 44% ± 9% (data not shown).

The inverted response to glucose seen in 50% of T2D islet preparations could be induced in ND islets by 2 μM diazoxide (Figure 7B), which increases K_ATP-channel activity by ~80 pS (Figure 3B). In the presence of diazoxide, 6 mM glucose stimulated rather than inhibited glucagon secretion. Similar results were observed in mouse islets (data not shown).

A common variant (E23K; rs5219) in KCNJ11 (which encodes the Kir6.2 subunit of the K_ATP-channel) is associated with enhanced T2D risk (Gloyn et al., 2003), increased K_ATP-channel activity (Schwanstecher et al., 2002), and impaired glucose-induced suppression of glucagon secretion in vivo (Tschröter et al., 2002). However, we found no difference in the inhibitory effect of glucose on glucagon secretion in vitro in ND human islets homozygous for the high-risk TT or low-risk CC variants (Figure 7C).
Treatment of ND mouse islets with oligomycin, a blocker of the mitochondrial ATP synthase, converted the inhibitory effect of glucose on glucagon secretion into stimulation (Figure 7 D). The ability of glucose to stimulate (rather than inhibit) glucagon secretion in islets treated with oligomycin suggests a regulatory role of nonmitochondrial metabolism in α cells. Interestingly, tolbutamide (10 μM) restored normal glucose regulation of glucagon secretion in islets from T2D donors (Figure 7 D) without correcting insulin secretion (data not shown). Collectively, these data suggest that in 50% of T2D patients, possibly because of a metabolic disturbance, KATP-channel activity is slightly increased with resultant loss of glucose-induced suppression of glucagon secretion. If this hypothesis is correct, then it should be possible to reverse the glucagon secretion defect in T2D with tolbutamide. Indeed, as shown in Figures 7 E and 7 F, tolbutamide (10 μM) partially restored glucose inhibition of glucagon secretion in islets from four T2D organ donors.

DISCUSSION

Our data show that pancreatic α cells respond to glucose with closure of KATP-channels and suggest that glucose modulates KATP-channel activity and glucagon secretion via increased cytoplasmic ATP/ADP (Figure 1 B). Although we favor the idea that α cells possess an intrinsic glucose sensing mechanism, we cannot rule out the involvement of the β cell metabolite GHB (γ-hydroxybutyrate), recently proposed to mediate the inhibitory effect on glucagon secretion (Li et al., 2013). The mechanism by which activation of the GHB receptor would inhibit glucagon secretion has not been established, but our data suggest it may culminate in KATP-channel closure.

Role of KATP Channels in Regulating α Cell Excitability

In most cells, K+ channel activity dampens cellular excitability by producing membrane hyperpolarization. Conversely, closure of K+ channels promotes electrical excitability. The β cell conforms to this principle: KATP-channel closure causes membrane depolarization, electrical activity, and insulin secretion. Strikingly, in α cells, KATP channel closure also causes depolarization but paradoxically inhibits glucagon secretion. Figure S5 illustrates how our data suggest this difference between α and β cells arises.

Crucially, in α cells, net KATP-channel activity at 1 mM glucose (50 pS; Figure 3 A) is only 0.5%–1.5% of the 3–9 nS measured under comparable conditions in β cells (Göpel et al., 200a;...
Zhang et al., 2008). As a result, α cells are electrically active at low glucose, stimulating glucagon secretion. Glucose elevation inhibits remaining $K_{ATP}$ channel activity, producing a further depolarization that partially inactivates voltage-dependent Na⁺ channels and decreases spike height. This reduces P/Q-type Ca²⁺-current activation and glucagon exocytosis. Although glucose also increases action potential frequency (+200%; Figure 2), this is insufficient to compensate for the reduced exocytosis (−75%; Figure 5A). The combination of these two effects can be estimated to result in ~50% inhibition of glucagon secretion, in good agreement with that observed experimentally (Figures 1A, 1C, and 1D).

The much larger $K_{ATP}$ conductance at 1 mM glucose keeps β cells hyperpolarized and electrically silent at low glucose, so that no insulin is secreted. Glucose closes $K_{ATP}$-channels and thereby triggers depolarization, electrical activity, and insulin secretion from a very low basal level. As in α cells, there is also a time-dependent reduction in spike height, but insulin secretion will remain much greater than in 1 mM glucose even after the reduction of spike height has occurred.

Our data suggest that tolbutamide and glucose affect electrical activity and glucagon secretion in freshly isolated islets by identical mechanisms. Very recently, glucose was postulated to inhibit glucagon secretion by a $K_{ATP}$-channel-independent (as yet unidentified) mechanism (Cheng-Xue et al., 2013), but the observation that the glucose-sensitive component of glucagon secretion was reduced by >85% in islets lacking $K_{ATP}$-channels argues that this mechanism is of relatively minor significance.

**Resting Activity of $K_{ATP}$ Channels Determines Islet Hormone Release**

The model outlined above postulates that the $K_{ATP}$-channel plays a dual role in the regulation of insulin and glucagon secretion. Depending on the initial activity, channel closure may either stimulate (β cells) or inhibit (α cells) secretion. Thus, increasing basal $K_{ATP}$ activity in α cells may result in a β cell phenotype—i.e., no electrical activity or glucagon secretion at 1 mM glucose, and stimulation of both by glucose. This is precisely what is seen in the presence of low concentrations of diazoxide (Figure 7B).

Similarly, we observed that glucose stimulates electrical activity in some α-V59M α cells (Figure 6C). Figure S6 illustrates the relationship between $K_{ATP}$-channel activity and glucagon secretion. Cells with little or no expression of mutant channels behave like control cells (Figure S6A). Cells expressing high levels of mutant channels (or exposed to high concentrations of diazoxide) will be permanently hyperpolarized and show no glucagon secretion at either 1 or 6 mM glucose (Figure S6B). Cells lying between these extremes will show a spectrum of responses: in some, 6 mM glucose will stimulate glucagon secretion (Figure S6C); in others, glucagon release will be high and unaffected by glucose (Figure S6D). It is of interest that the whole-cell $K_{ATP}$ conductance is ~3-fold larger in isolated rat α cells (Olsen et al., 2005) than that we observe in intact mouse islets. This may account for the paradoxical glucose-induced stimulation of glucagon secretion observed in single α cells.

Recently it was reported that the impaired counterregulation of glucagon secretion in diabetic islets could be restored by a somatostatin receptor antagonist (Yue et al., 2012), suggesting that somatostatin signaling is enhanced in diabetic islets. The effects of somatostatin on α cells include activation of K⁺-channels (GIRK) (Kailey et al., 2013). The resulting increase in GIRK-channel activity could be envisaged to have the same effect on glucagon secretion as a low concentration of diazoxide or weak expression of mutant $K_{ATP}$-channels.

Unexpectedly, glucagon secretion is close to normal in α-V59M islets, although α cell electrical activity is strongly affected (Figures 6C and 6D). This is consistent with the mild metabolic phenotype of α-V59M mice (Figures S4A and S4B) and suggests that glucagon secretion is upregulated in the 20% of α cells that remain active. However, the response to adrenaline was reduced by >70% (Figure 6E) in α-V59M islets, a finding that may explain the impaired insulin tolerance of α-V59M mice (Figure S4C). The stimulatory effect of adrenaline on glucagon secretion involves electrical activity and requires influx of extracellular Ca²⁺ (De Marinis et al., 2010). Thus, it seems likely that in α cells expressing mutant $K_{ATP}$-channels, which are electrically silent, adrenaline will be without stimulatory effect. Collectively, these findings argue that the subset of α cells in α-V59M islets that remain electrically active operate close to their maximum secretory capacity already at 1 mM glucose, accounting for the smallness of the adrenaline effect. This is reminiscent of reports of near-normal glucagon secretion in islets after almost total (98%) ablation of α cells by diphtheria toxin (Thorel et al., 2011). If this hypothesis is correct, then glucagon secretion evoked by experimental paradigms that bypass action potential firing should be enhanced in α-V59M islets. Indeed, high-[K⁺]o depolarization (which stimulates secretion in both electrically active and electrically silent α cells) produces a much larger stimulation of glucagon secretion in α-V59M than in control islets (Figure 6F).

It may seem surprising that glucose remains capable of inhibiting glucagon secretion in α-V59M islets despite no detectable glucose-induced reduction of $K_{ATP}$-channel activity. However, for the reasons we outlined above, glucagon secretion will principally reflect the activity of the small subset of cells in which $K_{ATP}$-channel activity is normal. By contrast, measurements of $K_{ATP}$-channel activity will be dominated by α cells expressing high levels of mutant $K_{ATP}$ channels, which respond poorly to glucose. Importantly, in some α cells, expression of mutant $K_{ATP}$-channels is low and total $K_{ATP}$-channel activity only marginally increased, just sufficient to suppress electrical activity at 1 mM glucose. However, when glucose is elevated and $K_{ATP}$-channel activity is reduced, these α cells undergo depolarization and start firing action potential with resultant stimulation of glucagon secretion. This opposes the glucose-induced inhibition in other cells and explains the reduced glucose-induced inhibition of glucagon secretion in α-V59M mutant islets.

In general, the subtle impact of targeting the Kir6.2-V59M mutation to the α cell on glucagon secretion suggests that enhanced α cell $K_{ATP}$-channel activity is unlikely to affect glucagon secretion in patients with neonatal diabetes due to gain-of-function $K_{ATP}$-channel mutations.

**Implications for Type 2 Diabetes**

In T2D, glucagon secretion in vivo is often stimulated rather than inhibited during hyperglycemia (Dunning et al., 2005). We observed a similar abnormality in 50% of islet preparations
from T2D organ donors (Figure 7A); the remaining preparations exhibited normal glucose regulation. Thus, T2D islets are heterogeneous with respect to the dysregulation of glucagon secretion, in agreement with a recent report (Li et al., 2013). Intriguingly, the inverted response to glucose seen in some T2D islet preparations can be mimicked in ND islets by a small increase (0.5%) in $K_{\text{ATP}}$-channel activity produced by diazoxide (Figure 7B). Similarly, metabolic inhibition, which activates $K_{\text{ATP}}$-channels, affects glucagon secretion in the same way as T2D (Figure 7D). This suggests that T2D may be associated with increased $K_{\text{ATP}}$-channel activity, possibly as a consequence of impaired islet metabolism (Dolba et al., 2012). It is notable that glucagon secretion in the presence of diazoxide or oligomycin shows not only an inverted glucose response but also reduced secretion at 1 mM glucose. This is reminiscent of the impaired counterregulation of glucagon secretion. It is therefore of interest that a low concentration of the $K_{\text{ATP}}$-channel blocker tolbutamide restores normal glucose regulation of glucagon secretion in metabolically compromised islets (Figure 7D) and improves it in islets from donors with T2D (Figures 7E and 7F). Possibly, low-dose sulfonylurea (much less than required to stimulate insulin secretion) may be a useful addition to insulin therapy. Finally, our data suggest that a single cellular disturbance (impaired glucose metabolism and ATP production), via increased $K_{\text{ATP}}$-channel activity, may explain the trio of hormone secretion defects associated with T2D: impaired glucose-induced insulin secretion, inverted glucose regulation of glucagon secretion, and defective counterregulation.

**EXPERIMENTAL PROCEDURES**

All experiments were conducted in accordance with the UK Animals Scientific Procedures Act (1986) and University of Oxford ethical guidelines.

**Media**

Media used are specified in Table S1.

**Animals and Generation of α-V59M Mice**

Most experiments were performed on islets isolated from NMRI mice obtained from a commercial supplier. Mice expressing Kir6.2-V59M in α cells (α-V59M mice) were generated using a Cre-lox approach (Clark et al., 2010).

**Human Islets**

Human islets (obtained with ethical approval and clinical consent) were isolated from pancreases of 46 nondiabetic donors and 10 donors with T2D.

**Genotyping of Human Islet DNA**

The rs5219 variant was genotyped using an allelic discrimination assay-by-design method on an ABI 7900 analyzer (Applied Biosystems).

**Hormone Release Measurements**

Measurements of insulin and glucagon secretion were performed using the in situ pancreas perfusion or static incubations of isolated islets.

**Electrophysiology**

All electrophysiological measurements were performed at +34°C on α cells within intact islets (from NMRI or α-V59M and control mice).

For the membrane potential and whole-cell $K_{\text{ATP}}$-current recordings (Figures 2A–2C), the perforated patch technique was employed as reported previously (De Marinis et al., 2010). The pipette solution consisted of IC1, and the bath contained EC2.

Exocytosis was measured as increases in membrane capacitance in α cells in intact islets as described previously (Göpel et al., 2004) using pipette medium IC2 and extracellular medium EC3. The impact of glucose on exocytosis was tested using the perforated patch technique using pipette medium IC3.

Voltage-dependent inactivation of the Na⁺-current was evaluated using the standard whole-cell technique and a two-pulse protocol using intra- and extracellular media IC4 and EC4, respectively.

**ATP Imaging**

The ATP/ADP sensor Perceval (Berg et al., 2009) was used as previously described (Tarasov et al., 2012).

**Identification of α Cells**

The identity of the α cells was established either by (1) immunocytochemistry following injection of the cell with biocytin (0.5 mg/ml) via the recording electrode (Figures S2C and S2D) or (2), in the case of α-V59M and control α cells, by tdRFP fluorescence. In perforated patch measurements of electrical activity and $K_{\text{ATP}}$-channel activity in NMRI islets (Figures 2–4), it was not always possible to identify the cell by immunocytochemistry because the cell detached when retracting the recording electrode. In these cases, α cells were identified by their spontaneous action potential firing at 1 mM glucose (Göpel et al., 2000a).

**Statistical Analysis**

Details on the analysis of the electrophysiological data are given in the Supplemental Information. Data are presented as mean values ± SEM of the indicated number of experiments (n). Error bars in figures represent SEM. Statistical significances were, unless otherwise indicated, evaluated using Student’s t test.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2013.10.014.

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