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The roles of voltage-gated potassium channels Kv2.1 and Kv2.2 in the regulation of insulin and somatostatin release from pancreatic islets

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Running Title: Distinct roles of Kv2.1 and Kv2.2 in islets

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Abbreviations

K_V channel, voltage-gated potassium channel; GSIS, glucose-stimulated insulin secretion; guangxitoxin-1E, GxTX-1E; Roswell Park Memorial Institute medium, RPMI medium; Krebs/Ringer buffer, KRB; FCS, fetal calf serum; small hairpin RNA, shRNA; somatostatin receptor subtype 5, SSTR5; intraperitoneal glucose tolerance test, IPGTT; Vitamin E d-alpha tocopheryl polyethyleneglycol succinate, VitE-TPGS; enzyme immunoassay, EIA; radioimmunoassay, RIA; bovine serum albumin, BSA; glucagon-like peptide-1, GLP-1; G-protein-coupled receptor 40, GPR40; dimethylsulfoxide, DMSO.

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Abstract

The voltage-gated potassium channels Kv2.1 and Kv2.2 are highly expressed in pancreatic islets, yet their contribution to islet hormone secretion are not fully understood. Here we investigate the role of Kv2 channels in pancreatic islets using a combination of genetic and pharmacological approaches. Pancreatic β-cells from $Kv2.1^{-/-}$ mice possess reduced Kv current and display greater glucose-stimulated insulin secretion (GSIS) relative to wild-type β-cells. Inhibition of Kv2.x channels with selective peptidyl (GxTX-1E) or small molecule (RY796) inhibitors enhances GSIS in isolated wild-type mouse and human islets, but not in islets from $Kv2.1^{-/-}$ mice. However, in wild-type mice neither inhibitor improved glucose tolerance *in vivo*. GxTX-1E and RY796 enhanced somatostatin release in isolated human and mouse islets and *in situ* perfused pancreata from wild-type and $Kv2.1^{-/-}$ mice. Kv2.2 silencing in mouse islets by adenovirus-shRNA specifically enhanced islet somatostatin, but not insulin, secretion. In mice lacking somatostatin receptor 5, GxTX-1E stimulated insulin secretion and improved glucose tolerance. Collectively, these data show that Kv2.1 regulates insulin secretion in β-cells and Kv2.2 modulates somatostatin release in δ-cells. Development of selective Kv2.1 inhibitors without cross inhibition of Kv2.2 may provide new avenues to promote GSIS for the treatment of type 2 diabetes.

Introduction

Pancreatic islets contain several types of highly interactive endocrine cells secreting specific hormones including insulin (β-cells), glucagon (α-cells), and somatostatin (δ-cells) (Muoio and Newgard, 2008; van Belle et al., 2011). Hormone release is tightly controlled by ion channels that regulate cell membrane potential and calcium influx (Rorsman, 1997; Kanno et al., 2002; Drews et al., 2010). Both members of the Kv2 voltage-gated potassium (Kv) channel family, Kv2.1 and Kv2.2, are expressed in pancreatic islets across several species (MacDonald et al., 2002, 2003; Yan et al., 2004; Herrington et al., 2005), with Kv2.1 being highly enriched in islet β-cells (MacDonald et al., 2002, 2003; Yan et al., 2004). Although germline null mutation of Kv2.1 has been shown to promote islet insulin secretion *in vitro* and to lower blood glucose levels *in vivo* (Jacobson et al., 2007), pharmacological studies with Kv2 inhibitors have not convincingly shown a role for Kv2 channels in regulating glucose stimulated insulin secretion (GSIS) in islets (Herrington et al., 2006; Braun et al., 2008), and, moreover the biological role(s) of Kv2.2 in islets has not been assigned.

Kv channels are also expressed in somatostatin-secreting islet δ-cells (Göpel et al., 2000; Braun et al., 2009), although it remains unclear whether they mediate δ-cell membrane repolarization and glucose-stimulated somatostatin secretion, similar to their roles in β-cells (Braun et al., 2009). In this study, with the use of selective Kv2.x inhibitors and genetic tools, we present evidence supporting the idea that Kv2.1 plays an important role in regulating insulin secretion in β-cells, whereas Kv2.2 is critical for the control of somatostatin secretion in δ-cells. The increase in somatostatin levels caused by Kv2.x inhibitors provides a negative feedback on the β cell that opposes the inhibitory

effect of these agents on Kv2.1 channels to increase insulin release and lower blood glucose. Our studies suggest that development of selective Kv2.1 inhibitors may provide new avenues to promote GSIS for the treatment of type 2 diabetes.

Methods

Compounds. Guangxitoxin-1E (GxTX-1E) was purchased from Peptides International (Louisville, KY). RY796 was synthesized at Merck Research Laboratories. All other reagents were purchased from Sigma-Aldrich Chemicals (St Louis, MO) unless otherwise specified.

Animals. Wild-type C57BL/6 mice were purchased from Taconic Farm (Germantown, NY). Kv2.1$^{-/-}$ mice (originally from Deltagen) were backcrossed onto a C57BL/6 background for 6 generations at Taconic Farm. Kv2.1$^{-/-}$ mice were produced through het x het breeding, and littermate wild-type were used as controls. Somatostatin receptor 5 knockout (SSTR5$^{-/-}$) mice, kindly provided by Dr. FC Brunicardi of Baylor College of Medicine (Houston, TX; Wang et al., 2005), were backcrossed and bred using a scheme similar to Kv2.1$^{-/-}$ mice. Mice used in this study were 2-6-month old, age- and gender-matched littermates including both sexes. All mice were acclimated in our laboratory for at least one week prior to experiments, and were given free access to rodent normal chow (Teklad 7012) and water and housed on a 12-h light/dark cycle. All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee of Merck & Co.

Isolation of pancreatic islets and the static GSIS assay. Mouse islets were isolated by collagenase digestion and discontinuous Ficoll gradient separation (Lacy, 1967) and cultured overnight in RPMI 1640 medium with 11 mM glucose. Insulin secretion was

determined by 1-h static incubation in 96-well format as previously described (Herrington et al., 2006).

Insulin secretion in perifused mouse and human islets. Two independent batches of human islets (purity >85%, viability >90%) from normal subject cadaver organ donors (aged 20 and 58 years old, respectively) were obtained from the Islet Cell Resource Centers and the National Disease Resource Interchange (NDRI, Philadelphia, PA) with appropriate consent and Merck review board approval for research use of human islets. Islets were hand-picked and transferred to RPMI medium (10% FCS, 5.5 mM glucose), and cultured for at least 24-h before use. Islets from wild-type (WT) and Kv2.1$^{-/-}$(*Kcnb1*$^{-/-}$*)* mice were also given an overnight recovery from the isolation in RPMI 1640 medium (10% FCS, 11 mM glucose). Insulin secretion was measured with an islet perifusion system as described previously (Herrington et al., 2006; Tan et al., 2008).

Kv2.2 silencing in mouse islets. A recombinant adenovirus containing a shRNA sequence specific to mouse Kv2.2 (Ad-shKv2.2, sequence information available upon request) was constructed by previously described methods (Bain et al., 2004). Mouse islets were treated with 4.15 x 10^8 pfu Ad-shKv2.2 48 hours prior to static insulin and somatostatin secretion assays, and TaqMan analysis of Kv2.2 mRNA levels.

Intraperitoneal Glucose Tolerance Test (IPGTT). IPGTT was performed with mice (n=5-7/group) fasted for 5-6 hours, as previously described (Tan et al., 2008). GxTX-1E (0.1, 1 and 10 mg/kg, i.p. injection) or RY796 (50 mg/kg, oral gavage) was administrated 30 min

prior to glucose challenge (dextrose 2 g/kg, i.p. injection) with their correspondent vehicle control (phosphate-buffered saline for GxTX-1E and 20% VitE-TPGS for RY796). In experiments designed to measure plasma hormones, blood samples were harvested via retro-orbital bleeding 20 min after glucose injection, followed by insulin, glucagon or somatostatin measurements by ELISA kits (ALPCO diagnostics).

Pancreas perfusion for insulin and somatostatin secretion measurement. An *in situ* pancreas perfusion model was used to measure insulin and somatostatin *ex vivo* (Zhou et al., 1999). Briefly, aorta and hepatic portal vein were cannulated and mouse pancreas was perfused at 1 ml/min with modified Krebs/Ringer buffer (KRB). Buffers and the perfusion chamber were saturated with an O_2/CO_2 gas mixture and maintained at 37°C. Fractions were collected every minute and assayed for somatostatin by EIA for both SS-14 and SS-28 (Phoenix Peptide, Burlingame, CA) or insulin by RIA (Linco Research, St. Charles, MO).

Electrophysiology. Primary islet cells were dispersed, plated on glass coverslips and cultured for up to 3 days before the patch-clamp studies (Herrington et al., 2006). Kv currents were recorded as described previously (Herrington et al., 2005, 2006). The standard pipette solution was (in mM) 120 KCl, 20 KF, 10 EGTA, 10 HEPES, 2 MgATP, pH adjusted to 7.2 with KOH. The standard external solution consisted of (in mM) 160 NaCl, 4.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 3 glucose, pH 7.2 with NaOH. BSA (0.1% wt:vol) was added to solutions containing GxTX-1E.

Statistics. Data are expressed as mean ± SE. Statistical analysis was conducted using either ANOVA followed by Newman-Keuls' post hoc test using Prism (Version 4.0.3, Graphpad, La Jolla, CA) or Student's t-test, as appropriate. Statistical significance was defined as two-tailed $P < 0.05$.

Results

Genetic silencing or pharmacological inhibition of Kv2.1 promotes insulin secretion from pancreatic β-cells.

Previous studies have suggested a role for Kv2.1 in the regulation of GSIS in pancreatic β-cells (MacDonald et al., 2002; Herrington et al., 2006; Jacobson et al., 2007). Consistent with a previous report (Jacobson et al., 2007), whole-cell patch-clamp studies in dispersed islet β-cells revealed ~70% reduction of Kv current in β-cells from Kv2.1$^{-/-}$ mice relative to those from wild-type (WT) littermates (257 ± 18 pA/pF in WT vs 81 ± 7 pA/pF in Kv2.1$^{-/-}$, Fig. 1A). In islet perifusion assays, GSIS elicited by 16 mM glucose was significantly enhanced in Kv2.1$^{-/-}$ islets compared to WT islets (Fig. 1B). In contrast, basal insulin secretion at 2 mM glucose was not altered by Kv2.1 deletion (Fig. 1B), consistent with the idea that Kv2.1 channels are only activated when the β-cell is depolarized and act to limit the amount of calcium that enters the cell to trigger insulin secretion.

GxTX-1E, a 36 amino acid peptide, selectively inhibits Kv2.1 and Kv2.2 channels with IC_{50} values of 1-3 nM (Herrington et al., 2006, Supplemental Fig. 1), and augments GSIS in mouse islets (Herrington et al., 2006). However, to date it has not been determined whether the effect of GxTX-1E in promoting GSIS is mediated through inhibition of Kv2.1, Kv2.2, or both channels. To that end, we first examined the effects of GxTX-1E on GSIS in islets from WT and Kv2.1$^{-/-}$ mice. Addition of GxTX-1E increased insulin secretion at 16 mM glucose by nearly 2-fold in WT islets (Fig. 1C). Compared to WT islets, Kv2.1$^{-/-}$ islets display greater insulin secretion in response to 16 mM glucose. However, addition of GxTX-1E did not cause any further increase of GSIS in Kv2.1$^{-/-}$

islets (Fig. 1C). In contrast, in both WT and Kv2.1$^{-/-}$ islets enhancement of GSIS by GLP-1 was indistinguishable, and the basal insulin secretion at 2 mM glucose was not affected by GxTX-1E in either type of islets (Fig. 1C). Thus, the promoting effect of GxTX-1E on mouse islet GSIS appears to be mediated primarily by Kv2.1.

From prior studies (Herrington et al., 2006, 2007), it is uncertain whether GxTX-1E or other Kv2.1 inhibitors are able to promote GSIS in human islets. We thus performed *in vitro* human islet perifusion assays, and assessed the effects of GxTX-1E on human islet GSIS. GxTX-1E profoundly enhanced insulin secretion when present in the perfusate during the glucose (16 mM) stimulation phase (Fig. 1D). The average levels of secreted insulin during the 16 mM glucose + GxTX-1E challenge (minutes 10-30) were nearly 3-fold (16.4 ± 1.5 μU/25 islets/min) greater than those after challenging with glucose-alone (5.8 ± 0.6 μU/25 islets/min). Once glucose was withdrawn from the perifusion system, secretion was quickly restored to basal levels (Fig. 1D). GxTX-1E did not alter potassium chloride (30 mM) stimulated insulin secretion tested after 10 min of washout, indicating that GxTX-1E did not have any prolonged action on either insulin secretion or islet insulin content (Fig. 1D). Whole-cell patch-clamp studies further demonstrated that GxTX-1E significantly suppressed Kv currents in dispersed human β-cells (Fig. 1E and 1F). Thus, the selective Kv2.x inhibitor GxTX-1E reduces human β-cell Kv currents and enhances GSIS, similar to its action in mouse β-cells (Fig. 1A and Herrington et al., 2006). Collectively, the data from genetic ablation of Kv2.1 and results with peptidyl Kv2.x inhibitors demonstrate that the Kv2.1 channel is an important target for enhancing GSIS in mouse and human pancreatic β-cells.

Small molecule inhibitor of Kv2.1 and Kv2.2 enhances GSIS in mouse and human islets

To develop additional, non-peptidyl, pharmacological tools for Kv2 channels, and to explore the chemical tractability of Kv2.1 for small molecule drug development, we screened a fraction of the Merck compound collection with an automated IonWorks™ electrophysiology assay using a cell line stably expressing human Kv2.1 (Herrington et al., 2011). Two primary hits were found to be selective for Kv2 channels over other Kv channels. Subsequent medicinal chemistry modifications resulted in the optimized small molecule Kv2 inhibitor RY796 (Fig. 2A). RY796 selectively inhibits Kv2.1 and Kv2.2 channels with IC_{50} values of 0.14 and 0.09 μM, respectively, without affecting the activities of a broad range of potassium, sodium and calcium channels (IC_{50} values > 10 μM, Herrington et al., 2011). Given the high degree of selectivity, we tested the effects of RY796 on GSIS in isolated mouse and human islets. Similar to GxTX-1E, RY796 significantly enhanced insulin secretion at 16 mM glucose in isolated islets from WT mice and humans (Fig. 2B, 2C). In addition, like GxTX-1E, RY796 did not alter GSIS in $Kv2.1^{-/-}$ islets (Fig. 2B). Thus, RY796 shares a similar mechanism to GxTX-1E in enhancing GSIS through inhibition of Kv2.1 channels in islet β-cells.

Kv2.x inhibitors do not reduce blood glucose levels in normal mice

Given their robust stimulatory effects on GSIS in cultured islets, we expected that GxTX-1E and RY796 would have strong *in vivo* insulinotropic effects and lower systemic blood glucose levels. Lean WT mice were dosed with GxTX-1E (by i.p. injection) or RY796 (by oral gavage) 20-30 min prior to an intraperitoneal glucose

tolerance test (IPGTT). Unexpectedly, GxTX-1E (dosed up to 10 mg/kg) had no significant effect on blood glucose during IPGTT in mice (Fig. 3A). Furthermore, the small molecule Kv2.x inhibitor (RY796) increased, rather than decreased, glucose levels of the IPGTT in lean mice relative to vehicle (20% Vitamin E d-alpha tocopheryl polyethyleneglycol succinate, VitE-TPGS) treated mice (Fig. 3B). By contrast, the small molecule GPR40 agonist (Cpd-C in Tan et al., 2008), a positive control for the study, significantly suppressed the glucose excursion during IPGTT (Fig. 3B). Thus, both Kv2.x inhibitors, GxTX-1E and RY796, failed to display blood glucose-lowering effects in mice *in vivo*.

The lack of glucose-lowering effect with GxTX-1E or RY796 did not result from insufficient pharmacokinetic exposure of the animals to the inhibitors. The plasma concentrations of GxTX-1E measured at the 60-min time point of the IPGTT were 2.5 μM in mice treated with 10 mg/kg GxTX-1E. This concentration of GxTX-1E is 800-fold above its IC_{50} value for inhibition of the cloned Kv2.1 channel (Herrington et al., 2006). The plasma level of RY796 (50 mg/kg) was 12.8 μM, which is 90-fold above its IC_{50} for Kv2.1 (Herrington et al., 2011). Serum protein binding of the agents, which is low for GxTX-1E (Ratliff et al., 2008) and moderate for RY796 (20-fold shift in 33% mouse serum) does not appear to be responsible for the lack of glucose-lowering efficacy. Given these results, we decided to investigate the reason(s) why Kv2.1 inhibitors failed to reduce glucose levels *in vivo* despite their dramatic enhancement of insulin release *in vitro*.

Kv2.x inhibitors augment circulating levels of somatostatin *in vivo*

To uncover these unknown mechanisms, we first examined the circulating levels of islet hormones, including insulin, glucagon, and somatostatin, in mice treated with a Kv2.x inhibitor, followed by a glucose challenge. WT and Kv2.1$^{-/-}$ mice were treated with GxTX-1E (10 mg/kg) 30 min prior to IPGTT, and blood samples were collected 20 min after glucose administration, followed by measurements of plasma insulin, glucagon, and somatostatin (SS-14/28) levels. Circulating plasma insulin and glucagon levels were not significantly altered by treatment with GxTX-1E (Fig. 3C, 3D). Similar to the experiment in Fig. 3A, basal blood glucose levels in mice treated with GxTX-1E were not different compared to vehicle controls (data not shown). However, levels of plasma somatostatin (SS-14/28) were significantly elevated in both WT and Kv2.1$^{-/-}$ mice treated with GxTX-1E (Fig. 3E). Similarly, *in vivo* administration of 50 mg/kg RY796 significantly augmented plasma SS-14/28 levels (190.6 ± 10.1 pg/ml for RY796 *vs.* 137.8 ± 3.6 pg/ml for vehicle, $p<0.05$, n=7), but not insulin (1.92 ± 0.35 ng/ml for RY796 vs. 2.05 ± 0.53 ng/ml for vehicle, n=7), or glucagon levels (98.5 ± 5.1 pg/ml for RY796 *vs.* 95.8 ± 5.8 pg/ml in vehicle, n=7). Thus, *in vivo* administration of Kv2.x inhibitors leads to an increase in circulating somatostatin.

Kv2.2 channels regulate somatostatin secretion from isolated islets

Pancreatic δ-cells of both rodent and human islets express similar ion channels and secretory machinery as β-cells (Göpel et al., 2000; Zhang et al., 2007; Braun et al., 2009). Importantly, pancreatic δ-cells are known to express Kv2.2 channels (Yan et al., 2004; Wolf-Goldberg, 2006). Since GxTX-1E (Herrington et al., 2006) and RY796 (Herrington et al., 2011) inhibit Kv2.2 channels with similar potency as Kv2.1 (Kv2.2

IC_{50} values of 3 nM and 90 nM, respectively), these inhibitors could be affecting δ-cells and β-cells concurrently in pancreatic islets. Thus, we directly measured glucose-stimulated somatostatin release from WT and Kv2.1$^{-/-}$ islets after exposure to these Kv2.x inhibitors. Compared to islets treated with DMSO, both inhibitors significantly augmented glucose-stimulated somatostatin secretion in WT and Kv2.1$^{-/-}$ islets (Fig. 4A). Basal somatostatin secretion or glucagon secretion was not altered in the presence of either of these Kv2.x inhibitors (Fig. 4A, 4B). Moreover, the stimulatory effects of GxTX-1E or RY796 on somatostatin release in WT and Kv2.1$^{-/-}$ islets were indistinguishable (Fig. 4A), suggesting that Kv2.1 is not involved in the enhanced somatostatin secretion observed in the presence of these Kv2.x inhibitors. These data support the notion that Kv2.2 channels play an important role in regulating somatostatin release from pancreatic δ-cells.

To test this possibility more specifically, we developed an adenoviral vector (Ad-shKv2.2) that carries a small-hairpin RNA specific for mouse Kv2.2 aimed to inhibit expression of Kv2.2 channels in intact isolated islets. The peripheral localization of the δ-cells in mouse islets should favor efficient delivery of the virus to these cells. Transduction of isolated mouse islets with Ad-shKv2.2 for 48-h led to ~ 65% reduction of Kv2.2 mRNA levels (Fig. 4C), and a significant enhancement of glucose-stimulated somatostatin release (Fig. 4D) with no effect on Kv2.1 mRNA expression (data not shown) or islet insulin secretion (Fig. 4E). Likewise, GxTX-1E also significantly enhanced glucose stimulated somatostatin and insulin secretion, but not glucagon release from cultured human islets (Fig. 5A-C). Taken together, our data suggest that Kv2.1

inhibition augments insulin secretion whereas Kv2.2 inhibition enhances somatostatin release from isolated pancreatic islets studied in vitro.

Intact pancreas architecture and circulation is required for paracrine inhibition of insulin secretion by somatostatin

Somatostatin is known to inhibit islet β-cell insulin secretion (Mazziotti et al., 2009). However, in cultured islets, treatment with Kv2.x inhibitors elevates β-cell GSIS despite a concomitant elevation in somatostatin secretion. This paradox could be due to a disruption of the normal islet architecture and circulation in isolated pancreatic islets, leading to an impaired paracrine regulation within the islet. We therefore performed *in situ* pancreas perfusion assays, an *ex vivo* system where the physiological δ-cell mass and native arterial vascular system are preserved. In contrast to what was observed in isolated islets, GxTX-1E did not promote insulin secretion following 16 mM glucose stimulation from either perfused WT or Kv2.1$^{-/-}$ pancreata (Fig. 6A,B). Kv2.1$^{-/-}$ pancreata did not manifest significant enhancement of insulin secretion at 16 mM glucose (Fig. 6A,B) compared to WT pancreata, possibly due to their reduced overall pancreas insulin content (Supplemental Fig. 2D). GxTX-1E augmented somatostatin release following 16 mM glucose stimulation from both WT and Kv2.1$^{-/-}$ pancreata (Fig. 6C,D). The perfused pancreas data are consistent with the *in vivo* data with Kv2.x inhibitors (Fig. 3), where somatostatin, but not insulin, is elevated. Collectively, our data imply that augmentation of somatostatin release via Kv2.2 inhibition has a powerful inhibitory action on the β-cells that may counteract the stimulatory effects of inhibiting Kv2.1 channels in β-cells.

Blocking somatostatin signaling unmasks Kv2.x inhibitor glucose lowering

Based on the findings in Fig. 6, we reasoned that blocking somatostatin signaling within the islet should lead to net enhancement of insulin secretion in the presence of Kv2.x channel inhibitors. Prior studies (Wang et al., 2005; Sprecher et al., 2010) have suggested that somatostatin suppresses insulin secretion mainly through binding to somatostatin receptor subtype 5 (Sstr5) expressed in pancreatic β-cells. Therefore, we studied SSTR5$^{-/-}$ mice lacking *Sstr5*. In perfused SSTR5$^{-/-}$ pancreata, GxTX-1E treatment dramatically increased GSIS (Fig. 7A). Furthermore, GxTX-1E significantly suppressed glucose excursion following glucose challenge in SSTR5$^{-/-}$ relative to SSTR5$^{-/-}$ mice treated with vehicle only (Fig. 7B), and augmented plasma insulin response to glucose challenge significantly in GxTX-1E-treated SSTR5$^{-/-}$ mice compared to SSTR5$^{-/-}$ mice treated with vehicle (Fig. 7C). Collectively, our data with GxTX-1E suggests that the positive effect of Kv2.1 inhibition in β-cells is likely canceled, at least in part, by stimulation of somatostatin secretion, due to inhibition of Kv2.2 channels in δ-cells.

Discussion

Peptidyl inhibitors of Kv2 channels, such as GxTX-1E, have provided strong evidence that these channels mediate the major voltage-gated K current in rodent pancreatic β-cells, and that their inhibition modulates GSIS (Herrington et al., 2006, 2007). In this study, we present pharmacological evidence that Kv2 channels play a similar role in the regulation of insulin secretion from human β-cells (Figs 1 & 2). In addition, the role of Kv2.1 and Kv2.2 channels in pancreatic islet hormone release has been elucidated through the use of both genetic and selective, peptidyl small molecule Kv2.x inhibitors. All data, taken together, support the idea that the effects of Kv2.x inhibitors on GSIS are mediated primarily by Kv2.1 since these agents have no effect on GSIS in islets lacking the Kv2.1 channel. The present data also suggest that the ability of the Kv2 inhibitors to enhance somatostatin secretion is due to inhibition of Kv2.2 channels present in δ-cells.

Despite the robust effects of Kv2.x inhibitors on islet GSIS *in vitro*, *in vivo* administration of GxTX-1E or RY796 did not improve glucose tolerance in the mouse. This lack of correlation between enhanced GSIS in isolated islets and glucose lowering efficacy *in vivo* prompted the study of these agents in the perfused pancreas model. The perfused pancreas model preserves the native islet mass and architecture and keeps the arterial vasculature of the pancreas intact. The present data suggest that the perfused pancreas model is a better predictor of *in vivo* GSIS efficacy than isolated islet GSIS. For example, inhibition of Kv2.1 channels led to an enhancement of GSIS in isolated islets, but this enhancement did not translate into an increase in insulin secretion from perfused pancreas, or to improved glucose tolerance *in vivo*. One possible explanation for the

divergent effects of Kv2 channel inhibition on GSIS in isolated islets versus perfused pancreas or *in vivo* is a difference in the paracrine regulation of the β-cells.

To explore possible paracrine interactions involved in the regulation of insulin secretion, the other major islet hormones (glucagon and somatostatin) were studied. No changes in glucagon were detected. To our surprise, a significant elevation of plasma somatostatin after glucose challenge *in vivo* and *in situ* (perfused pancreas) after GxTX-1E or RY796 treatment was observed. Since Kv2.2 is expressed in pancreatic δ-cells (Yan et al., 2004; Wolf-Goldberg et al., 2006), a role for Kv2.2 in regulating δ-cell somatostatin release was explored. We provide three lines of evidence that Kv2.2, but not Kv2.1, plays a major role in the regulation of δ-cell somatostatin secretion. First, ablation of Kv2.1 had no discernible consequences on somatostatin release *in vitro* or *in vivo*. Specifically, basal and glucose stimulated somatostatin secretion during IPGTT (Fig. 3E), in perfused pancreas (Fig. 4C and 4D), or in isolated islets (Fig. 5A) was not different between Kv2.1$^{-/-}$ and WT mice. Second, shRNA-mediated partial knockdown of Kv2.2 expression clearly enhanced somatostatin release in isolated mouse islets. Third, both peptidyl (GxTX-1E) and small molecule (RY796) Kv2.x inhibitors robustly enhanced glucose stimulated somatostatin secretion, even in the absence of Kv2.1.

Our understanding of the biology of the pancreatic δ-cells is limited, largely due to the difficulty in isolating these cells (Göpel et al., 2000; Zhang et al., 2007). Expression of Kv2.2 protein in δ-cells has been reported (Yan et al., 2004; Wolf-Goldberg et al., 2006), and δ-cells indeed have Kv currents (Göpel et al., 2000; Braun et al., 2009). However, the impact of inhibiting Kv2.2 on δ-cell physiology and somatostatin release had not been investigated in detail until the present study. Braun et

al. detected delayed rectifying Kv2 current in purified δ-cells, yet blocking the current with TEA or stromatoxin did not affect somatostatin release in intact human islets (Braun et al., 2009). In contrast, we find that Kv2 inhibition by GxTX-1E enhances somatostatin release in human islets (Fig. 5). The reason(s) for this discrepancy is not clear but may be explained by differences in the pharmacological agents used. TEA is a non-selective Kv channel blocker and inhibits other classes of channels as well. Also, although GxTX-1E is highly selective against other ion channels present in β-cells, the selectivity of stromatoxin for these channels, in particular voltage-gated calcium channels is not well defined. Further studies will be required to definitively address the role of Kv2.2 in insulin and somatostatin secretion.

The prominent role of Kv2.2 in the regulation of somatostatin release raises several questions for future studies. For example, is the lack of paracrine somatostatin action detected in the isolated islet GSIS assay due to loss of δ-cells from the periphery of the islet during the islet isolation procedure? Also, is the lack of *in vivo* glucose lowering seen with pan-Kv2 inhibitors due solely to paracrine somatostatin? The data on $SSTR5^{-/-}$ mice suggest somatostatin is the major factor preventing the *in vivo* efficacy of pan-Kv2 inhibitors. Since inhibition of Kv2.2 channels augmented somatostatin secretion from isolated islets and perfused pancreata, the major source of somatostatin is likely to be the δ-cells within the islet. However, our data do not exclude the possibility that systemically administered pan-Kv2 inhibitors may also cause release of somatostatin from another source, such as the gastrointestinal mucosa. Compared to the untreated cohorts, GxTX-1E significantly lowered the basal glucose level in $SSTR5^{-/-}$ mice, (Fig. 7B), suggesting that the inhibition of Kv2 channels may have additional effects on

peripheral tissues other than the pancreas. Our data also cannot exclude the possibility that pan-Kv2 inhibitors regulate Kv2.2 in cell types other than δ-cells in the pancreas.

Recently, Kv2.1 null ($Kv2.1^{-/-}$) mice have been reported to have reduced fasting blood glucose levels and elevated insulin secretion *in vivo* (Jacobson et al., 2007). In contrast, we did not observe any clear differences between $Kv2.1^{-/-}$ mice and their WT littermate controls in plasma glucose and insulin levels during an IPGTT (Supplemental Fig. 2). The Kv2.1 knockout mice used by Jacobson et al. (2007) and by us were re-derived from the same original line generated at Deltagen. The exact reason for the discrepancy between the glycemic phenotypes of the two sub-lines of $Kv2.1^{-/-}$ mice is not known. Our $Kv2.1^{-/-}$ mice weighed significantly less than their WT littermates and displayed improved peripheral insulin sensitivity (Supplemental Fig. 2). The insulin content of the $Kv2.1^{-/-}$ mouse pancreas was significantly reduced compared to WT littermate control (Supplemental Fig. 2D), suggesting reduced β-cell mass in this model. Given the marked divergence in body weight, insulin sensitivity and pancreatic insulin content between the WT and $Kv2.1^{-/-}$ mice, the utility of the $Kv2.1^{-/-}$ mouse model to discern the role played by Kv2.1 in whole body glucose metabolism is uncertain.

In summary, the results of the present study are consistent with a simple model where two closely related members of the Kv2 family play distinct roles in regulating pancreatic islet hormone release (Fig. 7D) in mice. In this model, Kv2.1 channels in the β-cell regulate GSIS, whereas in the δ-cell, Kv2.2 channels function to control the release of somatostatin. It is important to note that this model does not exclude the possibility that other Kv channels may also participate in the regulation of insulin secretion from the β-cell. Although the precise mechanism by which somatostatin attenuates insulin

secretion has not been explored in the present study, we speculate that somatostatin, by binding to specific receptors in β-cells, activates an inward rectifier potassium conductance that hyperpolarizes β-cells (Smith et al., 2001) and counteracts the action potential prolongation caused by Kv2.1 inhibition. Given that the interconnection between somatostatin and insulin release has been studied extensively in mice, it would be important to understand if somatostatin tone would also be a factor in limiting GSIS in humans when using a non-selective Kv2.x channel inhibitor. Given the importance of islet hormones in glucose homeostasis and the excellent track record of insulin secretagogues for successful blood glucose control in the clinic (Doyle and Egan 2003), development of a selective Kv2.1 inhibitor may provide a new avenue for the treatment of type 2 diabetes.

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Author Contributions:

Participated in research design: Li, Herrington, Eiermann, Jensen, Hohmeier, Newgard, Garcia, Zhang, Thornberry, Howard, Kaczorowski, Zhou

Conducted experiments: Li, Herrington, Petrov, Ge, Jensen, Hohmeier, Wagner

Contributed new reagents or analytic tools: Xiong

Performed data analysis: Li, Herrington, Petrov, Ge, Jensen, Hohmeier, Wagner, Zhou

Wrote or contributed to the writing on the manuscript: Li, Herrington, Newgard, Garcia, Kaczorowski, Zhou

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Footnotes

Conflict of interest: All authors, except MJ, HEH and CBN, are current or former employees of Merck & Co. and may hold stock or stock options in the company.

X.L. and J.H. contributed equally to this work.

Figure Legends

Fig. 1. Kv2.1 channel regulates glucose-stimulated insulin secretion in mouse and human islets. (A) Representative Kv currents in dispersed islet β-cells from WT and Kv2.1$^{-/-}$ mice. Currents were elicited by 100 msec depolarizing steps (in 10 mV increments) from a holding potential of -80 mV. **(B)** Insulin responses to glucose in islets from WT and Kv2.1$^{-/-}$ mice. Islets were perifused in parallel micro-chambers with KRB medium supplemented with 2 and 16 mM glucose as indicated. Data are mean ± SE of three independent experiments. **(C)** Basal and glucose stimulated insulin secretion in isolated WT and Kv2.1$^{-/-}$ islets after exposure to vehicle (DMSO), GxTX-1E (1 μM) or GLP-1 (50 nM), measured by static incubation assay. Data are mean ± SE of three independent experiments with at least 9 replicates in total. **$p<0.01$ vs. DMSO at 16 mM glucose in each group, $^{\#\#}p<0.01$ vs. WT islets after exposure to DMSO at 16 mM glucose. **(D)** GxTX-1E and GLP-1 enhance GSIS in human islets. Representative perfusion of human islets with KRB medium supplemented with glucose and potassium chloride (KCl) at the indicated concentrations and times. GxTX-1E (1 μM) or GLP-1 (10 nM) was added into the KRB medium during the course of 16 mM glucose perifusion. **(E)** Representative recording of Kv currents recorded in dispersed human islet β-cells without (control) or with 100 nM GxTX-1E treatment. **(F)** Percentage inhibition of Kv current by 100 nM GxTX-1E in CHO cells expressing human Kv2.1, and mouse and human islet β-cells. Data on recombinant Kv2.1 and mouse β-cells are taken from Herrington et al., 2006.

Fig. 2. Small molecule Kv2 inhibitor promotes GSIS in cultured mouse and human islets.

(**A**) Molecular structure of the selective Kv2 inhibitor, RY796. **(B)** Basal and glucose stimulated insulin secretion in cultured WT and Kv2.1$^{-/-}$ islets after exposure to vehicle (DMSO) or RY796 (10 μM), measured by static incubation assay. Data are means ± SE of three independent experiments with 9 replicates in total. **p<0.01, vs. DMSO (vehicle) at 16 mM glucose in each group. $^{\#}$p<0.05 vs. WT islets after exposure to DMSO at 16 mM glucose. **(C)** Basal and glucose stimulated insulin secretion in human islets after exposure to the indicated agents measured by static incubation assay. Data are means ± SE of two independent human islet donors with at least 6 replicates in total. *p<0.05 vs. DMSO (vehicle).

Fig. 3. Kv2 inhibitors fail to limit the glucose excursion in C57BL/6 mice during intraperitoneal glucose tolerance test (IPGTT). (A) IPGTT glucose profiles in mice treated with GxTX-1E (i.p. injection 20 min prior to glucose challenge). The area under the curve (AUC) of each group is shown on the right. **(B)** IPGTT glucose profiles in mice treated with indicated doses of RY796 or GPR40 agonist (oral gavage applied 30 min prior to glucose challenge. Data are means ± SE of 7-10 mice per group. *p<0.05, **p<0.01 vs. vehicle group. **(C-E)** Plasma hormone (insulin, **C**; glucagon, **D**; somatostatin, **E**) levels 20 min post glucose challenge in WT and Kv2.1$^{-/-}$ mice without (vehicle) or with GxTX-1E treatment before glucose challenge.

Fig. 4. Effects of inhibition of Kv2.1/Kv2.2 channels on somatostatin release from mouse islets. (A) Levels of somatostatin released from WT and Kv2.1$^{-/-}$ islets exposed to 16 mM glucose plus DMSO, or 1 μM GxTX-1E or 10 μM RY796 for 60 min. **(B)** Levels of glucagon released from WT mouse islets exposed to 16 mM glucose plus DMSO, or 1 μM GxTX-1E or 10 μM RY796 for 60 min. **(A-B)** Assays were performed in static incubation assay. Data are mean ± SE of three independent experiments with 9 replicates in total. **P<0.01 compared to vehicle controls in each case. **(C)** Relative mRNA levels of Kv2.2 in mouse islets 3 days post infection of sh-Kv2.2 or sh-control adenoviruses. **(D-E)** Basal and glucose stimulated somatostatin **(D)** and insulin **(E)** release from mouse islets 3 days post infection with sh-Kv2.2 or sh-control adenoviruses. **(C-E)** Data are mean ± SE of three independent experiments with 9 replicates in total. **P<0.01 compared to sh-control for each group.

Fig. 5. Kv2 inhibitor GxTX-1E enhances somatostatin and insulin release from cultured human islets. Levels of somatostatin **(A)**, insulin **(B)** and glucagon **(C)** released from cultured human islets exposed to 16 mM glucose plus DMSO, or 1 μM GxTX-1E or 50 nM GLP-1 for 60 min were measured by static incubation assay. Data are means ± SE of independent human islet donors with at least 6 replicates in total. *p<0.05 vs. DMSO (vehicle).

Fig. 6. Effects of GxTX-1E on insulin and somatostatin secretion in perfused pancreata from WT and Kv2.1$^{-/-}$ mice. Release of insulin **(A-B)** and somatostatin **(C-D)** in perifused pancreata from WT (**A** and **C**) or Kv2.1$^{-/-}$ (**B** and **D**) mice. Pancreata

were perifused at a rate of 3 ml/min with oxygenated KRB medium supplemented with glucose, at the indicated concentrations and time course, via cannulae placed into the aorta (influx) and portal vein (efflux) in a humidized chamber at 37ºC. Fractions of the perfusate were collected once a minute for insulin measurement. GxTX-1E (1 μM) was added only during the 30 min when glucose in the KRB was raised to 16 mM. Data are mean ± SE of three pancreata for each group.

Fig. 7. GxTX-1E stimulates insulin secretion and lowers blood glucose in SSTR5$^{-/-}$ mice. (A) Release of insulin in perifused pancreata from SSTR5$^{-/-}$ mice. Pancreata were perifused with KRB medium supplemented with glucose, at the indicated concentrations and times. GxTX-1E (1 μM) or vehicle was added during the 30 min when glucose in the KRB was raised to 16 mM. Data are mean ± SE of three pancreata for each group. **(B)** Blood glucose levels during an IPGTT in SSTR5$^{-/-}$ mice treated with vehicle or GxTX-1E (3 mg/kg, i.p.) 30 min prior to glucose challenge (dextrose 2 g/kg body weight, i.p.). The AUC of these two groups is shown on the right. **(C)** Plasma insulin levels in SSTR5$^{-/-}$ mice during IPGTT prior to and 10 min post glucose challenge. Data are mean ± SE, n=6-8 for each group. *$p<0.01$ compared to vehicle treated animals. **(D)** Illustration showing distinct roles of Kv2.1 and Kv2.2 in pancreatic islets. Kv2.1 primarily regulates GSIS from β-cells, whereas Kv2.2 regulates somatostatin release from δ-cells.

Figure 1

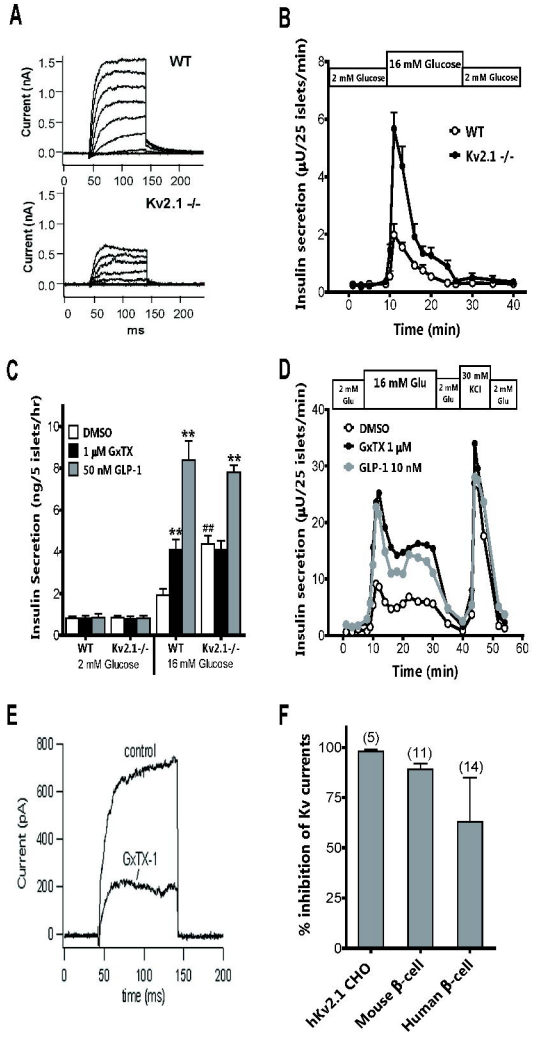

A
WT
Kv2.1 -/-
Current (nA)
ms
B
2 mM Glucose
16 mM Glucose
2 mM Glucose
WT
Kv2.1 -/-
Insulin secretion (µU/25 islets/min)
Time (min)
C
DMSO
1 µM GxTX
50 nM GLP-1
Insulin Secretion (ng/5 islets/hr)
WT
Kv2.1-/-
2 mM Glucose
16 mM Glucose
D
2 mM Glu
16 mM Glu
30 mM KCl
DMSO
GxTX 1 µM
GLP-1 10 nM
Insulin secretion (µU/25 islets/min)
Time (min)
E
control
GxTX-1
Current (pA)
time (ms)
F
% inhibition of Kv currents
(5)
(11)
(14)
hKv2.1 CHO
Mouse β-cell
Human β-cell

Figure 2

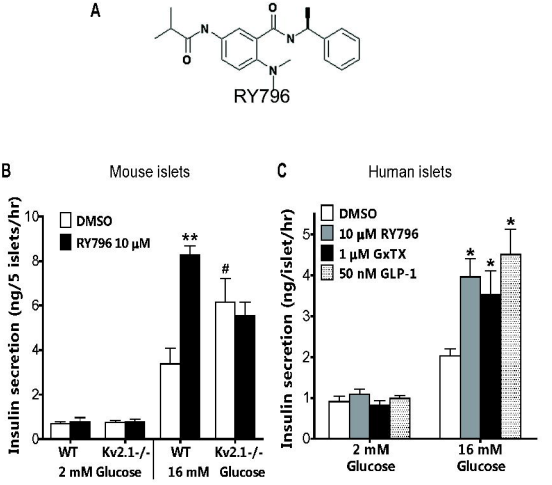

A
RY796
B
Mouse islets
Insulin secretion (ng/5 islets/hr)
DMSO
RY796 10 μM
**
#
WT
Kv2.1-/-
WT
Kv2.1-/-
2 mM Glucose
16 mM Glucose
C
Human islets
Insulin secretion (ng/islet/hr)
DMSO
10 μM RY796
1 μM GxTX
50 nM GLP-1
*
*
*
2 mM Glucose
16 mM Glucose

Figure 3

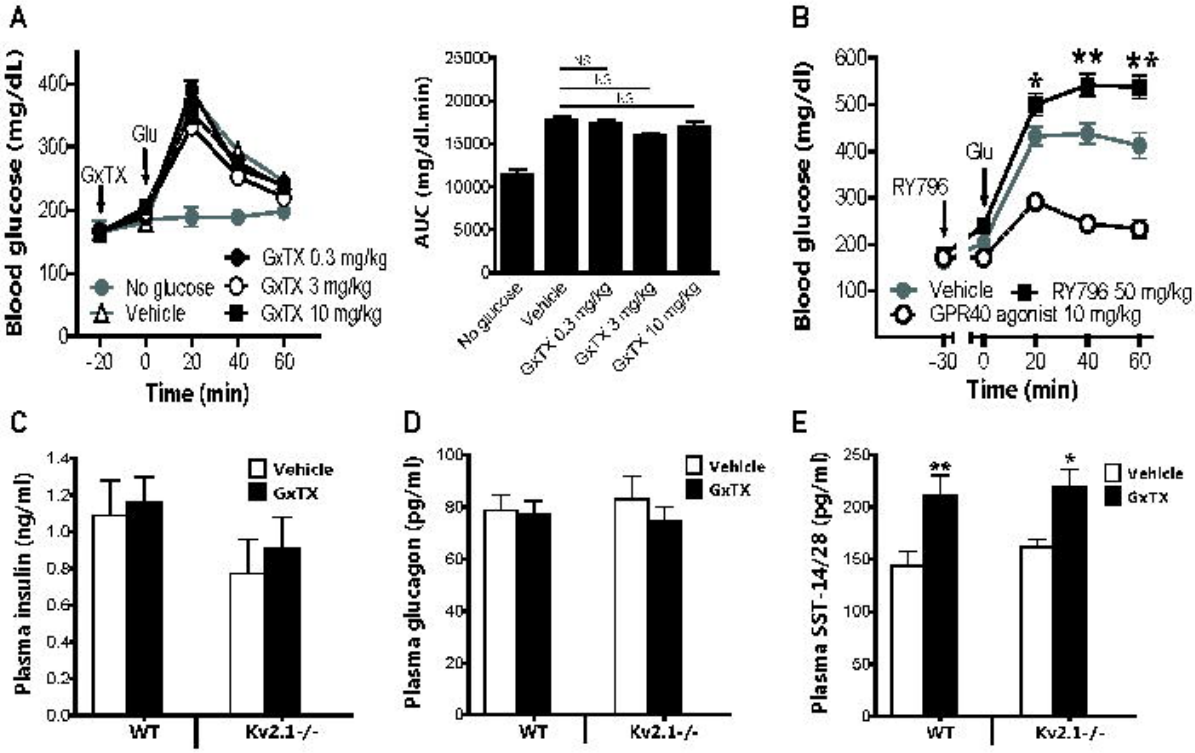

A
Blood glucose (mg/dL)
400
300
200
100
0
GxTX
Glu
No glucose
Vehicle
GxTX 0.3 mg/kg
GxTX 3 mg/kg
GxTX 10 mg/kg
-20
0
20
40
60
Time (min)
AUC (mg/dl.min)
25000
20000
15000
10000
5000
0
NS
NS
NS
No glucose
Vehicle
GxTX 0.3 mg/kg
GxTX 3 mg/kg
GxTX 10 mg/kg
B
Blood glucose (mg/dl)
600
500
400
300
200
100
RY796
Glu
*
**
**
Vehicle
RY796 50 mg/kg
GPR40 agonist 10 mg/kg
-30
0
20
40
60
Time (min)
C
Plasma insulin (ng/ml)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
GxTX
WT
Kv2.1-/-
D
Plasma glucagon (pg/ml)
100
80
60
40
20
0
Vehicle
GxTX
WT
Kv2.1-/-
E
Plasma SST-14/28 (pg/ml)
250
200
150
100
50
0
**
*
Vehicle
GxTX
WT
Kv2.1-/-

Figure 4

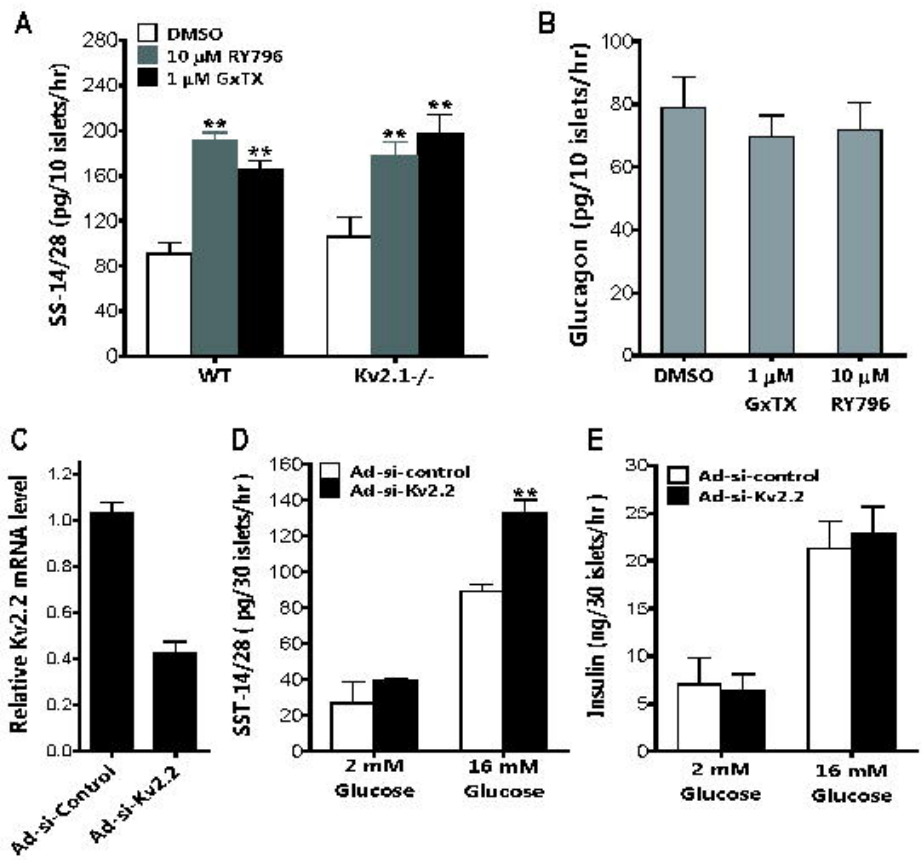

A
DMSO
10 μM RY796
1 μM GxTX
SS-14/28 (pg/10 islets/hr)
280
240
200
160
120
80
40
0
**
**
**
**
WT
Kv2.1-/-
B
Glucagon (pg/10 islets/hr)
100
80
60
40
20
0
DMSO
1 μM GxTX
10 μM RY796
C
Relative Kv2.2 mRNA level
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ad-si-Control
Ad-si-Kv2.2
D
Ad-si-control
Ad-si-Kv2.2
SST-14/28 (pg/30 islets/hr)
160
140
120
100
80
60
40
20
0
**
2 mM Glucose
16 mM Glucose
E
Ad-si-control
Ad-si-Kv2.2
Insulin (ng/30 islets/hr)
30
25
20
15
10
5
0
2 mM Glucose
16 mM Glucose

Figure 5

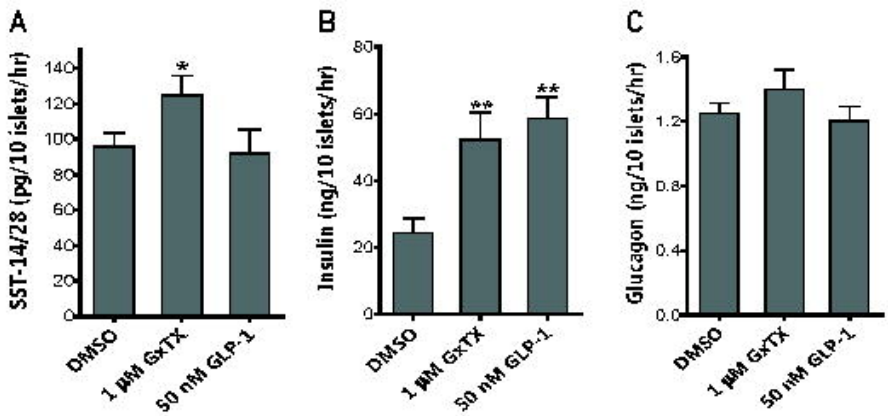

A
SST-14/28 (pg/10 islets/hr)
140
120
100
80
60
40
20
0
*
DMSO
1 μM GxTX
50 nM GLP-1
B
Insulin (ng/10 islets/hr)
80
60
40
20
0
**
**
DMSO
1 μM GxTX
50 nM GLP-1
C
Glucagon (ng/10 islets/hr)
1.6
1.2
0.8
0.4
0.0
DMSO
1 μM GxTX
50 nM GLP-1

Figure 6

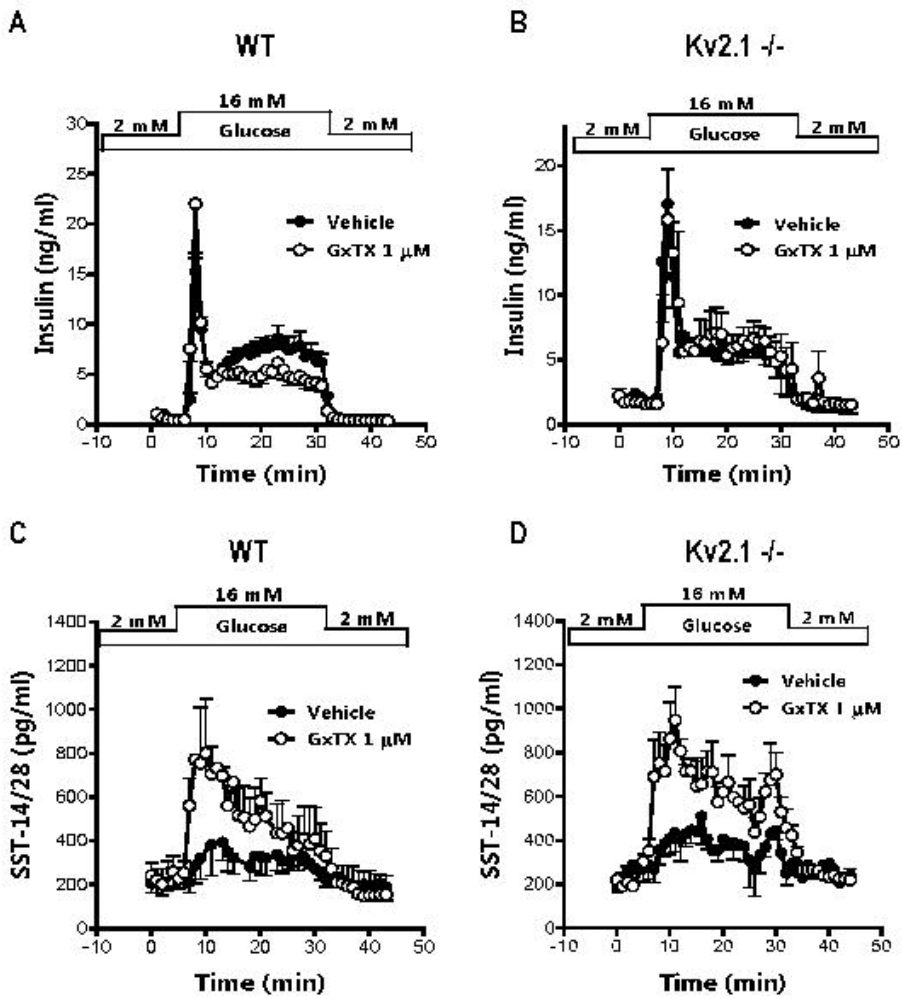
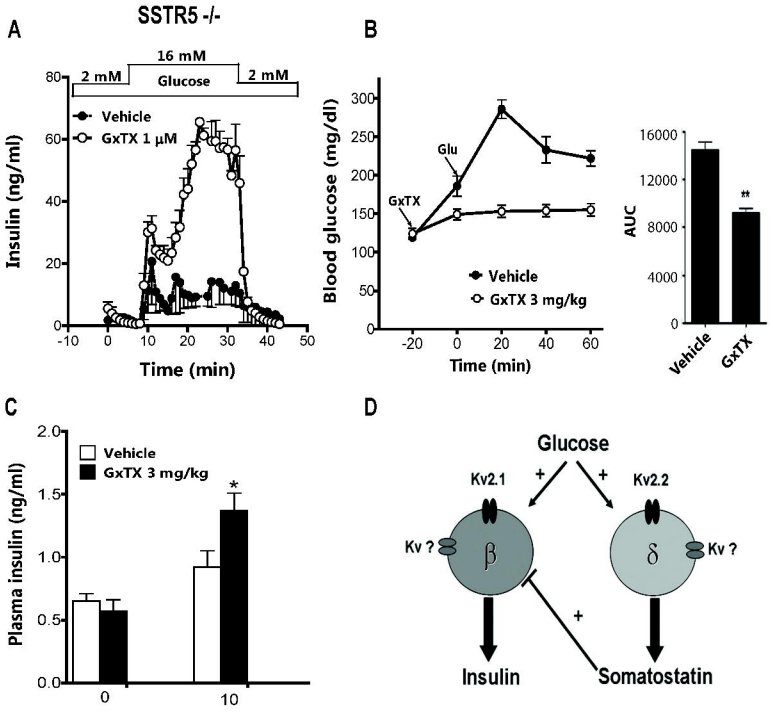

A
WT
16 mM
2 mM
Glucose
2 mM
Vehicle
GxTX 1 µM
Insulin (ng/ml)
Time (min)
B
Kv2.1 -/-
16 mM
2 mM
Glucose
2 mM
Vehicle
GxTX 1 µM
Insulin (ng/ml)
Time (min)
C
WT
16 mM
2 mM
Glucose
2 mM
Vehicle
GxTX 1 µM
SST-14/28 (pg/ml)
Time (min)
D
Kv2.1 -/-
16 mM
2 mM
Glucose
2 mM
Vehicle
GxTX 1 µM
SST-14/28 (pg/ml)
Time (min)

Figure 7


A
SSTR5 -/-
16 mM
2 mM
Glucose
2 mM
Vehicle
GxTX 1 µM
Insulin (ng/ml)
Time (min)
B
Blood glucose (mg/dl)
Glu
GxTX
Vehicle
GxTX 3 mg/kg
Time (min)
AUC
**
Vehicle
GxTX
C
Vehicle
GxTX 3 mg/kg
Plasma insulin (ng/ml)
*
D
Glucose
Kv2.1
Kv2.2
Kv ?
Kv ?
β
δ
Insulin
Somatostatin