Blockade of Na\textsuperscript{+} Channels in Pancreatic \(\alpha\)-Cells has Anti-Diabetic Effects

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Abstract

Pancreatic α-cells express voltage-gated Na\(^+\) channels (NaChs) which support the generation of electrical activity leading to increase in intracellular calcium and causes exocytosis of glucagon. Ranolazine, a NaCh blocker, is approved for treatment for angina. In addition to its anti-anginal effects, ranolazine has been shown to reduce HbA1c in patients with type 2 diabetes (T2DM) and coronary artery disease; however, the mechanism behind its anti-diabetic effect has been unclear. We tested the hypothesis that ranolazine exerts its anti-diabetic effects by inhibiting glucagon release via blockade of NaChs in the pancreatic α-cells. Our data show that ranolazine, via blockade of NaChs in pancreatic α-cells, inhibits their electrical activity and reduces glucagon release. We found that glucagon release in human pancreatic islets is mediated by the Na\(_{v}1.3\) isoform. In animal models of diabetes ranolazine and a more selective NaCh blocker (GS-458967) lowered postprandial and basal glucagon levels, which were associated with a reduction in hyperglycemia, confirming that glucose-lowering effects of ranolazine are due to blockade of NaChs. This mechanism of action is unique in that no other approved anti-diabetic drugs act via this mechanism, and raises the prospect that selective Na\(_{v}1.3\) blockers may constitute a novel approach for the treatment of diabetes.
**Introduction**

Glucose homeostasis is regulated primarily by the opposing actions of insulin and glucagon (1). Glucagon secreted by pancreatic α-cells regulates glucose homeostasis by promoting hepatic glucose production. Plasma glucagon levels are increased under both fasting and postprandial states in diabetes (2, 3). This hyperglucagonemia increases hepatic glucose production, thereby contributing importantly to diabetic hyperglycemia (4, 5). Therefore, not surprisingly, lowering of glucagon levels or antagonizing its actions via blockade of glucagon receptors can significantly reduce hyperglycemia (6, 7).

Regulation of glucagon secretion from pancreatic α-cells is modulated by the autonomic nervous system, gut hormones, paracrine factors and various ion channels. Amongst these various contributors, secretion of glucagon from α-cells depends on the generation of Na⁺-dependent action potentials (8). It is known that α-cells express Tetrodotoxin (TTX)-sensitive NaCh isoforms and NaCh blockers like TTX inhibits glucagon secretion (8-10). Recently, it was reported that pancreatic α-cells of diabetic mice, with chronic hyperglucagonemia, had increased Na⁺ current (I_{Na}), action potential duration, amplitude and firing frequency and increased glucagon content which primes the cells for increased glucagon release (11).

Ranolazine is an anti-anginal drug with cardioprotective properties (12). The proposed mechanism of its anti-ischemic effects is inhibition of late Na current due to blockade of the cardiac isoform of NaCh, Na_v1.5 (13). In addition, ranolazine has been shown to have anti-diabetic effects in clinical and non-clinical studies (14-18). Data from the CARISA study showed
that ranolazine, in a dose-dependent manner, lowered glycated hemoglobin (HbA1c) in patients with chronic angina and T2DM (14). Data from the MERLIN-TIMI-36 study demonstrated that ranolazine lowered HbA1c in diabetics and reduced the incidence of newly elevated HbA1c in initially normoglycemic patients (15, 16). In non-clinical studies, ranolazine was found to lower fasting and non-fasting glucose levels and preserve pancreatic β-cells in streptozotocin (STZ)-treated mice and Zucker diabetic fatty (ZDF) rats (17, 18) however, the mechanism(s) underlying the improvement in glycemia was not elucidated in these studies. In the present study we tested the hypothesis that anti-diabetic effects of ranolazine may be due to inhibition of glucagon secretion from pancreatic islets via blockade of NaChs in α-cells.

Results of the present study show that NaCh blockers inhibit glucagon secretion by blocking the Na\textsubscript{v}1.3 isoform of pancreatic α-cells which leads to glucagon and glucose lowering effects in animal models of diabetes. Given the role of I\textsubscript{Na} and Na\textsuperscript{+}-dependent action potentials of pancreatic α-cells in the secretion of glucagon, Na\textsubscript{v}1.3 selective NaCh blockers may provide a novel mechanism for the treatment of hyperglycemia.
Research Design and Methods

Animals and Animal care

All animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee of Gilead Sciences. Sprague Dawley (SD) rats, Zucker Diabetic Fatty (ZDF), and lean control rats were purchased from Charles River Laboratories (Hollister, CA and Wilmington, MA). Animals were housed on a 12h light/dark cycle at 22-25˚C with ad libitum access to food and water.

Reagents and Drugs

Unless noted, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Ranolazine and GS-458967 (triazolopyridine derivative, 6-(4-(trifluoromethoxy)phenyl)-3-(trifluoromethyl)-[1,2,4]triazolo[4,3-a]pyridine, (19)) were synthesized by Gilead Sciences. TTX was purchased from Tocris Bioscience (Minneapolis, MN).

Culture and Treatment of Human and Rat Pancreatic Islets

Pancreatic islets were isolated from male SD rats (300-400g) using Liberase TL (Roche Diagnostics, Dallas, TX) and Ficoll gradients (Mediatech, Inc., Manassas, VA) (20). Adult human pancreatic islets were obtained from donors of either sex age ranging between 32 -65 years, with BMI ranging between 21-41 (National Disease Research Interchange (Philadelphia, PA) or Prodo Laboratories, Inc (Irvine, CA)). Islets were cultured in islet culture medium (RPMI1640 containing 10% FBS, 11mM glucose, 100U/ml penicillin, 100µg/ml streptomycin,
2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate) for 2-7 days. Equal sized islets were transferred to a 96-well plate with 10 islets per well and then treated with various agents in 150µl of Krebs-Ringer buffer (in mM) (129 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 5 NaHCO₃, and 10 HEPES, pH7.4) with 0.1% BSA and 3mM glucose for 1h. Supernatants were collected and stored at -80°C until analysis.

**Dispersion and Transfection of Pancreatic Islet Cells**

Cultured islets were dispersed by accutase and the cell suspension was filtered through a 40µm cell strainer and washed once with DPBS-4% BSA. Islet cells were resuspended in Opti-MEM I and transfected with 50nM siRNAs (Santa Cruz Biotechnology, Dallas, TX) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). A portion of transfected cells was collected 48h later for qPCR. Another portion was seeded in a Laminin/Poly-D-Lysine coated 96-well plate (BD Biosciences, San Jose, CA) at 1.5×10⁴ cells/well and cultured for 48h for glucagon secretion experiments.

**Quantitative real-time RTPCR**

Total RNA was extracted using TRIzol reagent (Life Technologies). cDNA was synthesized using an iScript Reverse Transcription kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed using SYBR Green PCR Master Mix reagent (Life Technologies) on a Stratagene Mx3000P qPCR system (Agilent Technologies, Santa Clara, CA). The expression level of a gene was normalized relative to β-actin. Primers for α-subunits of human sodium channel
isoforms were designed with Beacon Designer 7.0 (Premier Biosoft, Palo Alto, CA) and sequences are shown in supplemental Table 1.

**Electrophysiology**

Rat islets were dispersed as above, plated on PDL/Laminin coated coverslips (BD Biocoat, BD Biosciences) and transfected with a rat glucagon promoter-eGFP plasmid to identify α-cells (21). Where indicated, α-cells were also co-transfected with either Scn3a targeted or control siRNA (Life Technologies, Carlsbad, CA). Membrane potential (Vm) and peak Na\(^+\) current (I\(_{Na}\)) were recorded 48-72h after dispersion using the perforated patch configuration, 200B Axopatch amplifier and pClamp 10.2. Cells without spontaneous action potentials were excluded from analysis and all compounds/drugs were dissolved in the bath solution.

Recordings of membrane potential were made at 32°C using the current-clamp configuration. The bath solution contained (in mM): 140 NaCl, 5 HEPES, 3.6 KCl, 2 NaHCO\(_3\), 0.5 NaH\(_2\)PO\(_4\), 0.5 MgSO\(_4\), 2.6 CaCl\(_2\), 10 dextrose, 10 sucrose, pH 7.35. Pipettes (3.5-5.0 MΩ) were pulled from borosilicate glass and tip-filled with an internal solution consisting of (in mM): 76 K\(_2\)SO\(_4\), 10 KCl, 10 NaCl, 5 HEPES, 1 MgCl\(_2\), pH 7.35 and Amphotericin B (0.3mg/mL). Series resistance (Rs) was monitored using a voltage step from -70mV to 0mV (5ms, 0.5Hz) and was allowed to stabilize prior to beginning the experiment (Rs < 30MOhm). Recordings (30s) were analyzed using the event detection (threshold of 10mV) to quantitate total charge movement (Area), resting membrane potential, action potential amplitude and action potential frequency. I\(_{Na}\)
recordings were made at room temperature in the voltage-clamp configuration. The $I_{Na}$-bath solution contained (in mM): 130 NaCl, 5 HEPES, 3.6 KCl, 2 NaHCO$_3$, 0.5 NaH$_2$PO$_4$, 0.5 MgSO$_4$, 2.6 CaCl$_2$, 3 dextrose, 20 TEA-Cl, 10 4-AP, 2.5 CoCl$_2$, 0.5 tolbutamide, pH 7.35. Leak currents were subtracted using an online P/4 procedure and series resistance was compensated. $I_{Na}$ averaged over multiple sweeps was analyzed before and after application of the compound or drug.

**Studies of ZDF rats**

Male ZDF and lean rats (5 weeks old) were acclimatized for 1 week. Drugs were given to the animals in Purina 5008 diet for 10 weeks at doses of $\sim$ 170mg/kg/d of ranolazine, 0.6mg/kg/d of GS-458967, and 30mg/kg/d of sitagliptin (Discovery Fine Chemicals, Dorset, UK). This dose of ranolazine yielded plasma concentrations between 8-10 $\mu$M which are slightly higher than the therapeutic concentration (6-8 $\mu$M). The dose of GS-458967 was selected to yield plasma concentration of 1-2 $\mu$M which is close to the IC$_{50}$ value for inhibition of Na$_v$1.3 (0.6 $\mu$M). Blood samples were collected via retro-orbital bleed under non-fasting and fasting (4-6h) conditions with addition of aprotinin (Thermo Fisher Scientific, Pittsburgh, PA).

**Studies of streptozotocin diabetic rats**

Diabetes was induced in SD rats using a single ip injection of streptozotocin (STZ, 65mg/kg). Fasting plasma glucose (FPG) and glucagon levels were measured before and 2 weeks after STZ injection. Diabetic and control SD rats were randomized to different groups for the oral glucose
tolerance test (OGTT). After an overnight fast, each group was treated with ranolazine (30mg/kg, po), GS-458967 (0.5mg/kg, po) or vehicle at -15min followed by a glucose load (2g/kg in water) at 0 min. Blood samples were obtained via retro-orbital bleed.

**Hypoglycemia induced glucagon release in SD rats**

After an overnight fast, male SD rats (12 week old) were dosed with vehicle, ranolazine (30 mg/kg, po at -15 min), glibenclamide (5mg/kg, po, at -15min), or GS-458967 (0.5mg/kg, po, at -60min) followed by insulin infusion (5mU/min, iv) for 60 min.

**Biochemical Measurements**

Glucose and HbA1c were measured by clinical chemistry analyzer (Olympus AU400). Glucagon levels were measured by an ELISA kit (R&D Systems, Inc, Minneapolis, MN). The sensitivity of the glucagon assay is: 14.7 pg/ml, reading range is 31.2 - 2,000 pg/ml. Intra assay variation (Coefficient of Variability: CV%) is 2.7-3.6 and inter-assay variation (CV%) is 5.8-8.9.

**Immunohistochemistry**

Three pancreatic sections from 6 ZDF rats per group were analyzed. The sections were immunostained with primary antibodies (insulin and glucagon) followed by secondary antibodies (FITC, Jackson ImmunoResearch West Grove, PA). Stained sections were photographed under a fluorescent microscope and quantitated using ImageJ software (NIH, Bethesda, MD).

**Statistics and Data Analysis**
All results are mean±SE. Data were analyzed using Graphpad Prism7 or OriginPro7. Differences among groups were analyzed using a Student’s t-test or 1-way or 2-way analysis of variance (ANOVA) followed by appropriate post-hoc tests. A p value less than 0.05 was considered statistically significant.
Results

NaCh blockers inhibit release of glucagon from isolated pancreatic islets

The effect of NaCh blockers on glucagon secretion was determined in human and rat pancreatic islets. Ranolazine, GS-458967 (a novel and selective NaCh blocker (19), and TTX (a broad spectrum NaCh blocker) significantly reduced glucagon secretion in a concentration-dependent manner in both rat and human islets (Figure 1A-C, G, H). Compared to the control, maximal reductions of glucagon secretion were observed with ranolazine at 10 µM (25±3%), GS-458967 at 3 µM (51±9%), and TTX at 0.1 µM (64±3%) in human islets. In rat islets ranolazine decreased glucagon secretion by 36±6% at 10 µM.

In contrast, veratridine, a NaCh activator (opener) increased glucagon secretion in human pancreatic islets in a concentration-dependent manner (data not shown), with a 10-fold increase at 30 µM (Figure 1D-F). Ranolazine, GS-458967 and TTX significantly reduced the veratridine (30 µM)-induced increase in glucagon secretion in a concentration-dependent manner (Figure 1D-F). Glucagon secretion was reduced by 36±11, 71±10 and 79±8% with ranolazine (10 µM), GS-458967 (3 µM), and TTX (0.1 µM), respectively. In rat islets, glucagon secretion was reduced by 69±11, 85±7 and 83±12% with ranolazine (10 µM), GS-458967 (1 µM), and TTX (30 nM), respectively (Figure 1H). Insulin secretion was not affected by these NaCh blockers under these conditions (supplemental Figure 1).

Na, 1.3 is the NaCh isoform in α-cells responsible for glucagon release
Gene expression levels of various isoforms of NaChs in human and rat pancreatic islets were determined by quantitative polymerase chain reaction (qPCR). Data show that Na\textsubscript{v}1.3 (SCN3A) and Na\textsubscript{v}1.7 (SCN9A) are the two major isoforms expressed in human pancreatic islets, followed by the Na\textsubscript{v}1.2 (SCN2A) and Na\textsubscript{v}1.6 (SCN8A) isoforms which are expressed at much lower levels (Figure 2A), whereas in rat islets the only major isoform expressed Na\textsubscript{v}1.3 (Scn3a) (Figure 2B).

Functional roles of Na\textsubscript{v}1.3 and Na\textsubscript{v}1.7 in glucagon secretion were determined by silencing the expression of these two isoforms using the small interference RNA (siRNA) approach in cells dispersed from human islets. Specific knockdown of Na\textsubscript{v}1.3 or Na\textsubscript{v}1.7 resulted in an average decrease of 66±6% and 56±2% gene expression of Na\textsubscript{v}1.3 or Na\textsubscript{v}1.7, respectively, compared to the control (Figure 2C). Knockdown of Na\textsubscript{v}1.3, but not Na\textsubscript{v}1.7, caused a significant reduction of veratridine-induced increase of glucagon secretion. Glucagon secretion induced by 10 and 30 \(\mu\)M concentrations of veratridine was attenuated by 98±3, 85±5, respectively, after knockdown of Na\textsubscript{v}1.3 in human islets (Figure 2D). These data suggest that Na\textsubscript{v}1.3 represents the functionally important isoform of NaChs that mediates glucagon secretion from pancreatic \(\alpha\)-cells.

**NaCh blockers inhibit Na\textsubscript{v}1.3 I\textsuperscript{Na} and electrical activity of isolated pancreatic \(\alpha\)-cells**

Ranolazine and GS-458967 reduced peak I\textsuperscript{Na} (no late component was observed) in \(\alpha\)-cells by 25±4% at 10 \(\mu\)M and 55±7% at 1 \(\mu\)M respectively, whereas 0.3 \(\mu\)M TTX caused a complete block which is consistent with selective expression of TTX-sensitive NaChs in \(\alpha\)-cells (Figure 3A and B). The spontaneous electrical activity of \(\alpha\)-cells was inhibited by 63±9% with 10 \(\mu\)M ranolazine, 64±5% with 1 \(\mu\)M GS-458967 and 89±4% with 0.3 \(\mu\)M TTX (Figure 3D, E).
Detailed analysis of the electrical activity revealed that NaCh inhibition predominately reduced the amplitude and frequency of the activity (Supplemental Figure 2C and 2E).

Knockdown of Na\textsubscript{v}1.3 with targeted siRNA in α-cell reduced peak I\textsubscript{Na} by 85% compared to a non-targeted siRNA control (-8±5 pA/pF versus -57±5 pA/pF, respectively, Figure 3C). Accordingly, reduction of I\textsubscript{Na} by knockdown of Na\textsubscript{v}1.3 caused a reduction of number of cells showing spontaneous electrical activity (0/5 cells) but not in non-targeted siRNA (3/5 cells).

Because we found that veratridine increases the release of glucagon (Figure 1), we investigated whether veratridine, which is known to increase I\textsubscript{Na}, also increases the electrical activity of α-cells. Figure 3F illustrates that 15 µM veratridine evoked an increase (9±2 fold) in α-cell electrical activity. In the presence of veratridine, inhibition of I\textsubscript{Na} with either ranolazine or GS-458967 was sufficient to nearly normalize α-cell electrical activity. The average inhibition of electrical activity was 71±11% for 10 µM ranolazine and 88±2% for 0.3 µM GS-458967 (Figure 3G). Veratridine evoked a small increase in the frequency of electrical activity, which was reversed by NaCh inhibition (Supplemental Figure 2F).

**NaCh blockers lower postprandial hyperglucagonemia**

The in-vivo glucagon lowering effects of ranolazine and GS-458967, were determined in diabetic (STZ-treated) rats. Two weeks after STZ injection, baseline glucagon and glucose levels were markedly elevated in STZ-treated rats compared with the untreated group (Figure 4A, C). In response to an oral glucose load, there was no increase in insulin levels in STZ diabetic rats (Figure 4E). Glucagon levels in the STZ+vehicle group (Figure 4A) were
significantly higher, peaking at 15 min (307±11 pg/mL), than the control group (130±10 pg/mL). Glucose levels were also significantly higher in the STZ+vehicle group (Figure 4C), peaking at 30 min at 751±16 mg/dL compared to 256±13 mg/dL in the control group. Both ranolazine and GS-458967, administered acutely, reduced the increase in glucagon levels in response to the oral glucose load, reaching significance at 15 min compared to the STZ+vehicle group (Figure 4A). Accordingly, the change in area under the curve (ΔAUC) for glucagon was significantly smaller in the ranolazine and GS-458967-treated groups as compared to the vehicle-treated group (Figure 4B). This decrease in glucagon levels was associated with significantly lower glucose concentrations (Figure 4C) at 15 and 30 min in compound-treated groups compared to the vehicle-treated group. Accordingly, the ΔAUC for glucose was also smaller in ranolazine and GS-458967-treated rats (Figure 4D). There was no effect of ranolazine or GS-458967 treatment on insulin levels (Figure 4E, F).

Glucose lowering effects of NaCh blockers in ZDF rats

Long-term treatment with ranolazine, GS-458967 or the dipeptidyl peptidase 4 inhibitor sitagliptin (used as a positive control) in diabetic ZDF rats reduced fasting glucose, glucagon and HbA1c levels. The vehicle-treated ZDF group had fasting hyperglycemia (from 105±5 at baseline to 341±12 mg/dL) and hyperglucagonemia (from 130±14 at baseline to 312±14 pg/mL) at 10 weeks. Ranolazine, GS-458967 and sitagliptin-treated groups had significantly lower fasting plasma glucose levels from week 4 to week 10 (Figure 5A) compared to the vehicle-treated group. Chronic treatment with ranolazine or GS-458967 also delayed the onset of
hyperglucagonemia during development of diabetes starting from week 4 to week 10. These effects of ranolazine and GS-458967 were comparable to that of sitagliptin (Figure 5B). HbA1c was 3.7-3.8% at baseline in the five groups and increased to 10.3±0.2% at 10 weeks in the vehicle-treated ZDF group. Consistent with the improvement in glucagon and glucose levels, HbA1c levels were significantly lower in the groups treated with ranolazine (6.3±0.4%), GS-458967 (6.2±0.5%) and sitagliptin (7.4±0.8%) than in the vehicle (10.2±0.2%) group at week 10 (Figure 5C).

The effect of NaCh blockers on islet morphology

Pancreatic islets from rats treated with ranolazine or GS-458967 showed more insulin and less glucagon staining compared to the vehicle-treated rats (Figure 5D). With the development of diabetes, the clear round boundary of the islets was destroyed in the vehicle-treated ZDF group as compared to the lean rats (Figure 5D). Relative to the lean rats, the insulin-positive area (91±2%) was less whereas the glucagon-positive area (156±18%) was significantly increased in the vehicle-treated ZDF rats (Figure 5E, F). Treatment with ranolazine or GS-458967 significantly increased insulin-positive cells (104±2%, 103±3%) and decreased glucagon-positive cells (70±12%, 79±23%), which led to a partial reversal of the abnormal ratio between the insulin- and glucagon-positive areas (vehicle: 4.7±1; ranolazine: 12±2; GS-458967: 12±3) (Figure 5G). These effects of ranolazine and GS-458967 were comparable to those of sitagliptin (10±2 ratio).

NaCh blockers do not suppress hypoglycemia-induced glucagon increase
To evaluate the potential for NaCh blockers to interfere with hypoglycemia-induced glucagon release, the effects of ranolazine and GS-458967 were studied during insulin-induced hypoglycemia in normal rats (Figure 6). As expected, glucagon levels in the vehicle-treated group increased significantly at 40 min (671±80 pg/mL) in response to hypoglycemia (Figure 6A, B). Treatment with ranolazine (Figure 6B) or GS-458967 (Figure 6C, D) did not suppress the increase in glucagon levels whereas glibenclamide (Figure 6B), an insulin secretagogue used in clinical practice, significantly decreased the hypoglycemia-induced glucagon response (331±57 pg/mL at 40 min). In addition, ranolazine and GS-458967 treatment did not affect the recovery from hypoglycemia, after stopping insulin infusion. The glibenclamide-treated group, on the other hand, showed a significant delay in recovery from hypoglycemia (Figure 6A).
Discussion

The present study elucidates the mechanism underlying the anti-diabetic effect of a NaCh blocker ranolazine. The main findings of the study are that 1) ranolazine, inhibits secretion of glucagon from human pancreatic islets; 2) the inhibition of glucagon secretion is due to the blockade of α-cell TTX-sensitive peak $I_{Na}$ and a concomitant decrease in electrical activity; 3) Na$_V$1.3 is the likely NaCh isoform in α-cells that mediates the glucagonostatic effect of NaCh blockers; and 4) treatment with ranolazine lowers glucagon and glucose levels in diabetic ZDF rats. Furthermore, another selective NaCh blocker GS-458967 has similar glucagon lowering effects in vitro and also has anti-diabetic effects in rodent models of diabetes.

NaChs modulate glucagon secretion

TTX-sensitive voltage-gated NaChs in pancreatic α-cells support the generation of electrical activity which increases intracellular calcium and causes exocytosis of glucagon (10, 22). All NaCh blockers inhibited the electrical activity of α-cells and reduced the release of glucagon in a concentration-dependent manner (Figure 1 and 2) whereas the NaCh activator, veratridine, enhanced electrical activity of α-cells and increased glucagon secretion from intact islets (Figure 1 and 3). These findings are consistent with previous reports demonstrating that NaChs can directly modulate glucagon secretion in intact islets obtained from non-diabetic rodents and humans (8-11, 22, 23). A recent report demonstrated no effect of TTX on glucagon release (24) however; the reason(s) for this discrepancy are not well-understood. Our data show that inhibition of glucagon secretion by NaCh blockers correlates with inhibition of $I_{Na}$. For example, ranolazine at 10 μM caused a 25% inhibition of $I_{Na}$ in α-cells and a 36% reduction in glucagon
release from rat islets. Although the role of α-cell dysfunction supporting increased glucagon release in diabetes has not been well-characterized, it was reported recently that α-cells from diabetic mice, with elevated circulating glucagon levels, have enhanced voltage-gated $I_{\text{Na}}$ density, increased action potential amplitude and firing frequency (11). These data suggest that an increase in $I_{\text{Na}}$ may be an important mechanism underlying the hypersecretion of glucagon from α-cells and imply that inhibition of $I_{\text{Na}}$ could restore normal glucose homeostasis in diabetes.

Ranolazine and GS-458967 did not affect insulin secretion (supplemental Figure 1) under conditions where glucagon inhibition was observed. Although a previous study with ranolazine showed an increase in insulin secretion under high glucose conditions (18), the mechanism of this effect is not well-understood at this time, but seems to be independent of NaCh blockade as GS-458967 does not have any effect on insulin secretion even under high glucose conditions.

Among the 9 NaCh isoforms present in the excitable cells/tissues, the NaCh isoforms expressed at the highest level in human pancreatic islets are Na,1.3 and Na,1.7. Of the two, the Na,1.3 seems to be functionally responsible for glucagon secretion, as knockdown of Na,1.3 gene expression caused a significant reduction in $I_{\text{Na}}$, electrical activity and glucagon release. Consistent with our findings, a recent report identified Na,1.3 as the isoform responsible for glucagon secretion in mouse islets (25). Ranolazine and GS-458967, which lack isoform selectivity, inhibit Na,1.3 with IC$_{50}$ values of 12 µM (26) and 0.6 µM, respectively, which is consistent with the data for the inhibition of glucagon release.
NaCh blockers reduce glucagon and glucose levels in vivo

Postprandial hyperglycemia and hyperglucagonemia are characteristics of patients with diabetes (1). Reducing the post-prandial paradoxical increase in glucagon response is a potential target for lowering postprandial glucose levels. Both ranolazine and GS-458967, given acutely, significantly reduced the glucagon response to glucose load in diabetic rats. This is the first demonstration of a selective NaCh blocker reducing the increase in glucagon levels during an oral glucose load in diabetic animals. The effect was similar to that reported with GLP-1 receptor agonists, which also inhibit glucagon secretion albeit via a different mechanism (27).

Chronic administration of ranolazine or GS-458967 significantly lowered glucagon, glucose and HbA1c levels in ZDF rats. Relative to the vehicle group, ZDF rats treated with ranolazine or GS-458967 for 10 weeks had more insulin and less glucagon producing cells. Even though the NaCh blockers prevented the increase in glucagon levels, glucose levels continued to increase with time suggesting that glucagon is only partially responsible for hyperglycemia in this model. At later time points, the improvement in β-cell mass observed may also contribute to the lowering of glucose levels in drug treated groups. The magnitude of these effects of ranolazine was comparable to that of sitagliptin. In clinical studies, ranolazine has been reported to lower HbA1c between 0.5-0.7% (14) which is similar in magnitude to what has been reported for sitagliptin.
Ranolazine and GS-458967 did not affect the hypoglycemia-induced secretion of glucagon or recovery from hypoglycemia (Figure 6). These data suggest that effect of ranolazine and GS-458967 on glucagon is unlikely to be mediated via inhibition of autonomic nervous system as it has been shown that a substantial portion of hypoglycemia induced increase in glucagon levels is mediated via activation of autonomic nervous system (28). This is consistent with the very low incidence of hypoglycemic events observed with ranolazine in clinical practice. In our study, 10 μM ranolazine reduced normal or veratridine-evoked glucagon release from pancreatic islets by 25-40%, respectively and 100 nM TTX reduced glucagon release by no more than 80%. These results suggest that additional depolarizing currents, such as calcium-mediated depolarization could prevent the complete suppression of glucagon secretion following I_{Na} inhibition. Therefore, the glucagonstatic actions of NaCh blockers are not expected to compromise the physiologic defense against hypoglycemia.

**Glucagon as a target for diabetes**

The abnormal glucagon secretion in T2DM and its key role in development of fasting and postprandial hyperglycemia has been the focus of attention recently (1, 7, 29). Therefore, suppression of glucagon secretion or inhibition of its action in the liver constitutes potential therapeutic targets for diabetes (30-32). Results of studies with glucagon receptor antagonists (peptide and small molecule), antisense oligonucleotides and glucagon receptor knock-out mice show that elimination or inhibition of glucagon receptor signaling has strong anti-hyperglycemic effects in various animal models of diabetes (31, 33-35). Significant dose-dependent reductions
in HbA1c have been reported with glucagon receptor antagonists MK-0893 (0.6 to 1.5%, compared to placebo +0.5) and LY-2409021 (0.7-1%) by in patients with T2DM (33, 36).

The glucose lowering effects of glucagon receptor antagonists are also associated with increases in glucagon levels, liver enzymes, LDL cholesterol and α-cell hypertrophy & hyperplasia (37, 38). Hyperglucagonemia, accompanied by hypertrophy and hyperplasia of the pancreatic α-cells has been observed in glucagon receptor knock-out mice (34) and mice treated with glucagon receptor antisense oligonucleotides (35). In contrast, in our study ZDF rats treated with NaCh blockers for 8 weeks had no change in lipid levels (supplemental Table 2), had lower circulating glucagon levels and fewer α-cells than vehicle-treated rats (Figure 5), a finding that is consistent with the results of our previous study (18). Together these data suggest that normalization of circulating glucagon levels by correcting abnormal glucagon secretion by α-cell may be a better approach than blocking glucagon receptor signaling in the liver for treatment of diabetes. It has been suggested that inappropriately high glucagon levels in diabetes are due to impairment in α-cell function (39). Furthermore, α-cells from diabetic mice have enhanced $I_{Na}$ density, increased action potential amplitude, and firing frequency with no change in $Ca^{2+}$ currents (11). The authors proposed that alterations in electrical properties of α-cells may prime the cells for enhanced glucagon secretion, with no change in $Ca^{2+}$ currents. These data suggest that an increase in $I_{Na}$ may be an important underlying mechanism for glucagon hypersecretion in diabetes and our results are consistent with this interpretation. This hypothesis is consistent with our data in that effect of ranolazine on glucagon secretion in normal islets and in normal animals
is modest to minimal (when NaCh function is normal) but the effect is enhanced when NaChs are activated by veratridine in islets or in diabetic animals. Although the findings in mouse α-cells need to be confirmed in human α-cells, the HbA1c lowering effect of ranolazine observed in clinical studies is also consistent with this hypothesis (14, 15). Furthermore, clinical data with ranolazine shows that the HbA1c lowering effect of ranolazine is greater in patients starting with higher HbA1c (16). Although glucagon levels in these studies were not measured, higher HbA1c levels indirectly suggest a greater impairment of β-cell function (i.e. insulin loss), resulting in hyperactivity of α-cells, which in turn leads to higher glucagon levels. In this context, ranolazine would be expected to be more effective in patients exhibiting abnormally elevated α-cell electrical activity and glucagon secretion.

**Clinical Implications**

The HbA1c lowering effect of ranolazine has been previously demonstrated in three clinical studies but the mechanism of this effect remained unclear. The present study shows that NaCh blockers inhibit glucagon release from pancreatic islets, and also have anti-diabetic effects due to direct inhibition of I_{Na} in α-cells. Overall, these data suggest that a major contributing factor to increased glucagon levels may lie at the α-cell level (hypersecretion of glucagon), which can be corrected by blockade of Na_{v}1.3 channels. Although the role of NaChs in the pathophysiology of human diabetes requires further investigation, the findings from the present study suggest that inhibition of α-cell I_{Na}, could become an attractive drug target for combination with other classes of anti-diabetic agents.
Acknowledgments

A.K.D. provided input for study design and interpretation of the data, and wrote the manuscript. M.Y., Y.N., K.K. and M.K. designed and carried out experiments, collected and analyzed data. S.R and L.B. contributed to the study design and interpretation of the data. All authors reviewed, edited and approved the manuscript. A.K.D. and S.R. are the guarantors of this work and take full responsibility for the integrity of the data and accuracy of the data analysis.

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Figure Legends

Figure 1: NaCh blockers reduce glucagon secretion in human (A-F) and rat (G-I) pancreatic islets in a concentration-dependent manner. Isolated islets were cultured for 2 to 7 days before experiments. Ten equal size islets per group were selected and incubated for 1 h under low (3 mM) glucose conditions with ranolazine (A and G), GS-458967 (B) or TTX (C), or in the presence of 30 µM veratridine with ranolazine (D and H), GS-458967 (or GS, E and I) or TTX (F and I) at the indicated concentrations. Data presented as mean±SE of % of values of the control (C) from 4-8 independent experiments where each experimental condition was run in triplicate. *) p<0.05, **) p<0.01 and ***) p<0.001 vs. vehicle in the upper panels and vs. veratridine (V) alone in the lower panels.

Figure 2: Nav1.3 is the NaCh isoform responsible for glucagon release in human and rat pancreatic alpha cells. (A) Gene expression levels of NaCh isoforms measured using qPCR from isolated islets of 10 human donors (A) and SD rats (n=6, each with 4 rats) (B). (C) Gene expression of Nav1.3 or Nav1.7 in dispersed islet cells transfected with siRNAs for control (scrambled), Nav1.3 or Nav1.7. Data are presented as mean ± SE of % of values in control group set as 100%. (D) Isolated transfected cells were treated with different concentrations of veratridine for 1 h. Data presented as mean±SE of % of values of the control group (C), which were set as 100% from 7 experiments where each experimental condition was run in triplicate. **) p<0.01 and ***) p<0.001 vs. control.
**Figure 3:** Inhibition of Na\(_{V}1.3\) I\(_{Na}\) reduces spontaneous and veratridine-evoked electrical activity of rat pancreatic α-cells. (A) Representative traces of I\(_{Na}\) recorded from dispersed alpha cells in control buffer, 10 µM ranolazine or 0.3 µM TTX. (B) Average inhibition of I\(_{Na}\) by ranolazine (n=8) and GS-458967 (n=5). (C) Targeted knockdown of α-cell Na\(_{V}1.3\) reduced I\(_{Na}\) expression. Representative traces of spontaneous electrical activity of alpha cell in the presence of control buffer or ranolazine (D). The bar denotes solution exchange. (E) Average inhibition of spontaneous electrical activity with either 10 µM ranolazine (n=5), 1 µM GS-458967 (n=6) or 0.3 µM TTX (n=9). (F) Representative traces of veratridine evoked increase in alpha cell electrical activity, which was reduced by 10 µM ranolazine followed by 0.3 µM TTX. The bars denote solution exchange. (H) Average inhibition of veratridine-evoked electrical activity observed for 10 µM ranolazine followed by 0.3 µM TTX (n=5) and 0.1 and 0.3 µM GS-458967 (n=4). Data are presented as mean±SE. *) p<0.05 and **) p<0.01 vs. control and †) p<0.01 vs. veratridine.

**Figure 4:** Ranolazine and GS-458967 lower postprandial hyperglucagonemia in STZ-treated diabetic rats. Rats were fasted over-night before the experiment. Healthy SD rats were used as normal controls. Vehicle, ranolazine (30 mg/kg) or GS-458967 (0.5 mg/kg) was given via an oral gavage at -15 min followed by oral glucose (2 g/kg) at 0 min. (A) Glucagon, (C) Glucose and (E) Insulin levels during OGTT in all groups. Changes in area under the curve (ΔAUC) during OGTT for glucagon (B), glucose (D) and insulin (F) were determined. Data are presented as
mean±SE. *) p<0.05 and **) p<0.01 vs. STZ + vehicle group; † (p<0.05) and †† (p<0.01) STZ+vehicle group vs. control+vehicle group.

**Figure 5:** Ranolazine and GS-458967 improve basal hyperglucagonemia and hyperglycemia in Zucker Diabetic Fatty (ZDF) diabetic rats. (A) Fasting plasma glucose (FPG), (B) Glucagon and (C) HbA1c were monitored through 10-week treatment. Data are presented as mean ± SE. *) p<0.05; **) p<0.01 vs. vehicle treated group. (D) Insulin (red) and glucagon (green) staining of representative pancreatic islets from lean rats and ZDF rats treated with ranolazine, GS-458967 or sitagliptin for 10 weeks. All sections from fluorescent staining were digitally photographed and 3 sections from each of 6 animals per treatment groups were analyzed for percentage of (E) insulin positive or (F) glucagon positive cells compared to ZDF lean rats and (G) insulin:glucagon ratio per islet. Data are presented as mean±SE. *) p<0.05 vs. vehicle treated ZDF group.

**Figure 6:** Ranolazine and GS-458967 do not suppress the hypoglycemia-induced glucagon increase in normal Sprague Dawley (SD) rats. (A) Glucose and (B) glucagon levels in normal SD rats subjected to hypoglycemia caused by insulin infusion in the presence of ranolazine (30 mg/kg, p.o.) or glibenclamide (5 mg/kg, po) given at -15 min before starting insulin infusion. (C) Glucose and (D) glucagon levels in rats treated with GS-458967 (0.5 mg/kg, po) dosed at -60 min in a similar study. Data are presented as mean±SE. *) p<0.05 and **) p<0.01 vs. vehicle-treated group.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Online-only Supplemental Material

For

Blockade of Na⁺ Channels in Pancreatic α-Cells has anti-diabetic effects

Arvinder K. Dhalla, Ming Yang*, Yun Ning*, Kristopher M. Kahlig*, Michael Krause, Sridharan Rajamani, and Luiz Belardinelli

Supplemental Figure 1. Effect of NaCh blockers on insulin secretion in human and rat pancreatic islets. Isolated human or rat islets were cultured for 2 to 7 days before experiments. Ten equal size islets per group were selected and incubated for 1 h under 3 mM glucose, supernatants were harvested for insulin measurement. The effect of ranolazine or GS-458967 at the indicated concentrations on human islets are shown in (A) and (B), respectively. The effects of 10 µM ranolazine or 1 µM GS-458967 on rat islets are shown in (C). Data presented as mean ± SE from 3-5 independent experiments where each experiment was run in triplicate.
**Supplemental Figure 2.** Inhibition of pancreatic α-cell $I_{\text{Na}}$ modulates spontaneous and veratridine-evoked electrical activity through the (A-B) resting membrane potential as well as electrical activity (C-D) amplitude and (E-F) frequency. Resting membrane potential, electrical activity amplitude and electrical activity frequency were normalized to the value recorded in the control condition. NaCh inhibition with ranolazine, GS-458967 and TTX significantly decreased the amplitude of the α-cell spontaneous electrical activity (panel C). Veratridine evoked a non-significant increase in the electrical activity frequency, which could be normalized by NaCh inhibition (panel F). These data are mean ± SE calculated from the cells reported in Figures 3E and 3G. (*) $p<0.05$ and (**) $p<0.01$ vs. control and †) $p<0.05$ vs. veratridine.
Supplemental Table 1: qPCR primer sequences

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Supplemental Table 2. The fasting insulin and lipid profile of ZDF rats treated with vehicle or ranolazine for 10 weeks. Male ZDF (6 weeks old, 7-8 rats per group) were treated with vehicle or ranolazine (~170 mg/kg/day) in Purina 5008 diet for 10 weeks. Insulin was measured using ELISA kit and lipid profile was measured by clinical chemistry analyzer (Olympus AU400). Data presented as mean ± SE.

<table>
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<th>Triglycerides (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
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<td>Ranolazine (n=8)</td>
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<td>164.6±7.6</td>
<td>527.9±70.4</td>
<td>7.5±0.9</td>
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