

膵 β 細胞 Kv2.1 チャンネルによるインスリン分泌制御機構の解明と
糖尿病治療展開

**Role of Kv2.1 channel in insulin secretion in pancreatic β -cells and
its blockade to treat type 2 diabetes**

博士課程

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ABSTRACT OF DISSERTATION

Voltage-dependent potassium channels are involved in repolarization of excitable cells. In pancreatic β -cells, activation of voltage-gate K^+ (Kv) channels possibly repolarizes cells and attenuates glucose-induced action potentials to suppress insulin secretion. Among Kv channel families, Kv2.1 is reportedly expressed in islet β -cells as the major component in rodents. It is expected that inhibition of the β -cell Kv current prolongs action potentials and enhances glucose-induced insulin secretion.

Glucagon-like peptide-1 (GLP-1)-based medicines have recently been widely used to treat type 2 diabetic patients, while adverse effects of nausea and vomiting have been documented. Inhibition of voltage-gated K^+ channel subtype Kv2.1 in pancreatic β -cells has been suggested to contribute to mild depolarization and promotion of insulin release. This study aimed to determine the effects of pharmacological or genetic blockade of Kv2.1 channels on the glucose-induced increases in insulin release and cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in islets and β -cells, respectively, in mice. Furthermore, this study aimed to determine whether blockade of Kv2.1 channels potentiates insulinotropic effect of GLP-1 agonists.

In islets isolated by collagenase digestion, guangxitoxin-1E (GxTx), a Kv2.1 channel blocker, significantly increased glucose (8.3 mM)-induced insulin release without altering basal insulin release at 2.8 mM glucose. Glucose-induced insulin release from isolated islets of Kv2.1^{+/-} mice was significantly greater than that of wild-type mice. Likewise, blockade of Kv2.1 channels by GxTx potentiated glucose (8.3 mM)-induced $[Ca^{2+}]_i$ increases in β -cells without altering basal $[Ca^{2+}]_i$ levels at 2.8 mM glucose, as monitored by fura-2 microfluorometry. These results suggest that Kv2.1

channels physiologically restrict glucose-induced Ca^{2+} influx and thereby attenuate insulin secretion in β -cells.

When Kv2.1 channel blocker guangxitoxin-1E (GxTx) and GLP-1 agonist exendin-4 at the concentrations ineffective on insulin release (sub-threshold doses) were added together, this combination markedly increased insulin release and $[\text{Ca}^{2+}]_i$ in a glucose-dependent manner in mouse islets and β -cells. In Kv2.1^{+/-} mice, exendin-4 at sub-threshold concentration alone was capable of increasing insulin release and $[\text{Ca}^{2+}]_i$ in islets and β -cells. The $[\text{Ca}^{2+}]_i$ response to sub-threshold exendin-4 and GxTx in combination was attenuated by pretreatment with protein kinase-A (PKA) inhibitor H-89, indicating PKA-dependency of the cooperative effect.

In *in vivo* study, sub-threshold doses of GxTx and GLP-1 agonist liraglutide in combination markedly increased plasma insulin and improved glucose tolerance in diabetic db/db mice and NSY mice. These results suggest a modest suppression of Kv2.1 channels dramatically raises insulintropic potency of GLP-1 agonists, which opens a new avenue to reduce the doses of GLP-1 agonist and associated adverse effects while achieving the same glycemic control in type 2 diabetes.

1 INTRODUCTION

Voltage-gated potassium channels (Kv channels) consist of four alpha subunits, each containing six transmembrane domains (S1-S6). These channels are the largest family among the potassium channel groups, which in humans consist of 40 genes and are divided into 12 subfamilies (Kv1 to Kv12). These channels repolarize membrane potential in excitable cells (1,2).

Glucose metabolism-induced closure of ATP-sensitive K^+ (K_{ATP}) channels and membrane depolarization open the voltage-dependent Ca^{2+} channels, triggering Ca^{2+} influx and insulin secretion. In parallel, glucose-induced depolarization activates Kv channel, thereby repolarizing β -cells (3-5). Kv2.1 channel is the major component of Kv currents in rodent. Kv2.1 encoded by KCNB1 is expressed in islet β -cells in rodents and humans (6-8). Studies with the pharmacological Kv2.1 blockers (6,9,10) and Kv2.1 knockout (KO) mice (11) have suggested that this channel physiologically limits glucose-induced insulin secretion in β -cells, although most of the pharmacological Kv2.1 channel blockers can also inhibit Kv2.2 (9,12). It was reported that the KCNB1 single-nucleotide polymorphism genotype is associated with an increased risk of type 2 diabetes in the Chinese Han population (13). Since the β -cell Kv channels are open only when β -cells are depolarized by glucose, blockade of Kv2.1 channels in β -cells could promote insulin release with a lower risk of hypoglycemic events compared to the K_{ATP} channel blockers.

Glucagon-like peptide-1 (GLP-1), a physiological incretin hormone, is secreted from the intestinal L cells in response to meals and enhances insulin release to maintain normoglycemia (14,15). Circulating GLP-1 is rapidly degraded by dipeptidyl peptidase-

4 (DPP-4) with a half-life around 1-2 minutes. Currently, chemically modified DPP-4-resistant GLP-1 agonists and DPP-4 inhibitors have been clinically used for treating type 2 diabetic patients, and shown to confer a much lesser risk of hypoglycemia than sulfonylureas. However, nausea and vomiting have been documented as the common adverse events of GLP-1 agonists (16,17). A practical way to reduce these side effects is to lower their doses. This could be achieved by combining other substance or mechanism that can collaborate with GLP-1 agonists on insulin release.

GLP-1 potentiates glucose-induced insulin release from pancreatic β -cells by increasing intracellular cyclic AMP (cAMP), whose major actions are promotion of distal secretory processes associated with exocytosis (18) and facilitation of Ca^{2+} influx through voltage-dependent L-type channels (19). In addition, GLP-1 reportedly attenuates β -cell Kv channels possibly via cAMP signaling (20). This mechanism is suggested to partly contribute to the prolonged depolarizing effect of GLP-1 (20). A method to effectively inhibit Kv channels is expected to enhance the GLP-1 action on islet β -cells and facilitate GLP-1-based therapy.

This study aimed to clarify:

1. Whether pharmacological or genetic blockade of Kv2.1 channels could enhance the glucose-induced $[\text{Ca}^{2+}]_i$ increases and insulin release in pancreatic islet β -cells.
2. Whether pharmacological or genetic blockade of Kv2.1 channels could enhance exendin-4-induced $[\text{Ca}^{2+}]_i$ increases and insulin release in islet β -cells.
3. Whether genetic and pharmacological blockade of Kv2.1 channel in combination with GLP-1 agonist could improve glycemia in glucose tolerance tests in type 2 diabetic animals.

2 MATERIALS AND METHODS

2.1 Animals

Wild-type C57BL/6J mice (Japan SLC, Hamamatsu, Japan), Kv2.1-knockout (KO) mice (11) (Deltagen, Inc., San Mateo, CA), db/db mice (Japan Clea, Tokyo, Japan) and NSY mice (Japan SLC) were purchased, and housed on a 12-hour light/dark cycle in accordance with our institutional guidelines and with the Japanese Physiological Society's guidelines for animal care. Kv2.1-KO mice were backcrossed onto a C57BL/6J mice at least for nine generations. The disrupted sequence in genomic DNA from the KO mice was detected using PCR to produce an amplicon with one primer inside the targeting sequence in combination with a Kv2.1-specific primer. Male age-matched (10 weeks-old) Kv2.1 hetero-KO (Kv2.1^{+/-}) mice and wild-type littermates as controls were used. There was no difference on body weight between wild-type mice and Kv2.1^{+/-} mice (24.24 ± 0.93 g and 24.73 ± 0.36 g, respectively, $n = 8$). All mice were given free access to rodent normal chow and water. Experimental protocols for animal studies were approved by Jichi Medical University Animal Care and Use Committee.

2.2 Preparation of pancreatic islets and single β -cells

Islets of Langerhans were isolated by collagenase digestion, as reported (21) with slight modification. Animals were anaesthetized by intraperitoneal injection of pentobarbitone at 80 mg/kg, followed by injection of collagenase at 1.05 mg/ml (Sigma-Aldrich, St. Louis, MO) into the common bile duct. Collagenase was dissolved in HEPES-added Krebs-Ringer bicarbonate buffer (HKRB) solution (in millimoles): NaCl

129, NaHCO₃ 5.0, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.0, MgSO₄ 1.2 and HEPES 10 at pH 7.4 with NaOH, supplemented with 5.6 mM glucose and 0.1% BSA. The HKRB solution containing 0.1 % BSA was used for the measurements of cytosolic Ca²⁺ concentration ([Ca²⁺]_i) and insulin release but not for the patch-clamps study. Pancreas was dissected out and incubated at 37°C for 16 minutes. Islets were hand collected under a microscope and were immediately used for the measurement of insulin secretion. For β-cell experiments, islets were dispersed into single cells in Ca²⁺-free HKRB, and the single cells were plated sparsely on coverslips and maintained for 1 day at 37°C in an atmosphere of 5% CO₂ and 95% air in Eagle's minimal essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin.

2.3 Measurements of insulin release in mouse islets

For measurements of insulin release, groups of 10 islets were incubated for 1 hour at 37°C in HKRB with 2.8 mM glucose for stabilization, followed by test incubation for 1 hour in HKRB with 2.8 or 8.3 mM glucose. Guanylin-1E (GxTx) (Peptide Institute, Osaka, Japan), exendin-4 (Ex-4) (Sigma-Aldrich), and GxTx with Ex-4 were present throughout the incubation. Insulin release in islets was determined by ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan).

2.4 Measurements of [Ca²⁺]_i in single β-cells

Dissociated single β-cells on coverslips were mounted in an open chamber and superfused in HKRB. Cytosolic Ca²⁺ concentrations ([Ca²⁺]_i) in single β-cells were measured at 31°C by dual-wavelength fura-2 microfluorometry with excitation at

340/380 nm and emission at 510 nm using a cooled charge-coupled device camera (21,23). The ratio image was produced on an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). Data were taken exclusively from the cells that fulfilled the reported morphological and physiological criteria of β -cells, including the diameter and responsiveness to glucose (8.3 mM) and K_{ATP} channel blocker tolbutamide (100 μ M). For $[Ca^{2+}]_i$ measurements, β -cells were prepared from at least three mice in each experiment.

2.5 Patch-clamp experiments in mouse single β -cells

Conventional whole-cell currents were recorded using an amplifier (Axopatch 200B; Molecular Devices, Foster, CA), in a computer using pCLAMP10.2 software, as reported (22). For conventional whole-cell clamp experiments, pipette solution contained (in millimoles): KCL 50, K_2SO_4 35, $MgCl_2$ 5, EGTA 11, $CaCl_2$ 1, HEPES 11 and ATP-2Na 5 at pH 7.2 with KOH. HKRB solution containing 5.6 mM glucose was used for external solution. Single β -cells were voltage-clamped at a holding potential of -70 mV and then shifted to the test potential to 0 mV with the pulses of 100 milliseconds duration for Kv channel currents at room temperature (25°C).

2.6 Intraperitoneal glucose tolerance tests and oral glucose tolerance tests

An intraperitoneal glucose tolerance tests (IPGTT) were performed with male $Kv2.1^{+/-}$ mice and wild-type littermates (10 weeks-old), db/db mice (7 weeks-old) or NSY mice (20 weeks-old) fasted overnight, as previously reported (24). One g/kg glucose into db/db mice or 2 kg/kg glucose into $Kv2.1^{+/-}$ mice, wild-type mice and NSY mice was injected intraperitoneally (i.p.), followed by blood sampling from the tail vein.

Saline (0.1 ml/10 g body weight), GxTx (100 nmol/kg, i.p.), liraglutide (Novo Nordisk) (3 nmol/kg, i.p.) or liraglutide with GxTx was administrated 30 minutes before the glucose challenge. Liraglutide at doses of 30 μ g/kg (~10 nmol/kg) and higher is reported to substantially reduce blood glucose in mice (25). Hence, in the present study, we used 3 nmol/kg as a sub-threshold dose. In oral glucose tolerance tests (OGTT), 2 g/kg glucose was orally injected into wild-type and Kv2.1^{+/-} mice. Blood glucose concentrations were measured using a GlucoCard DIA meter (Arkray, Kyoto, Japan), and insulin concentrations using an ELISA kit (Morinaga Institute of Biological Sciences).

2.7 Measurements of systolic blood pressure

Systolic blood pressure and heart rate were measured by tail-cuff method (Model MK-2000; Muromachi Kikai, Tokyo, Japan) (26). Male db/db mice (7 weeks-old) were habituated with measuring systolic blood pressure for 5 days prior to experiment. Saline (0.1 ml/10 g body weight), GxTx (100 nmol/kg, i.p.) or liraglutide (3 nmol/kg, i.p.) together with GxTx was administrated to the mice, and systolic blood pressure and heart rate were measured before and 30 min after the administration at 13:00 to 15:00 (light phase).

2.8 Statistical analysis

Data represent the means \pm s.e.m. Statistical analyses were performed using the unpaired or paired Student's *t*-test or a one-way ANOVA followed by Bonferroni multiple comparison tests. *P* values below 0.05 were considered statistically significant. A χ^2 test was used for comparison of incidence of $[Ca^{2+}]_i$ oscillation in β -cells.

3 RESULTS

3.1 Chapter I. Blockade of Kv2.1 channels enhances the glucose-induced $[Ca^{2+}]_i$ increases and insulin release in pancreatic islet β -cells

3.1.1 GxTx enhances glucose-induced $[Ca^{2+}]_i$ increases in β -cells of mice

To examine the effect of pharmacological blockade of Kv2.1 channel in pancreatic β -cells, I used Guanyxitoxin (GxTx), a potent inhibitor of Kv2.1 channel. GxTx (30 nM) did not have any effect on basal $[Ca^{2+}]_i$ at 2.8 mM glucose. A rise in glucose concentration from 2.8 to 8.3 mM increased $[Ca^{2+}]_i$ in mouse β -cells. After the glucose-induced first-phase $[Ca^{2+}]_i$ increases were ended, administration of GxTx (30 nM) for 3 minutes increased $[Ca^{2+}]_i$. Conversely, antidiabetic drugs sulphonylurea tolbutamide (0.1 mM), increased $[Ca^{2+}]_i$ both in the presence of 2.8 mM and 8.3 mM glucose (Fig.1).

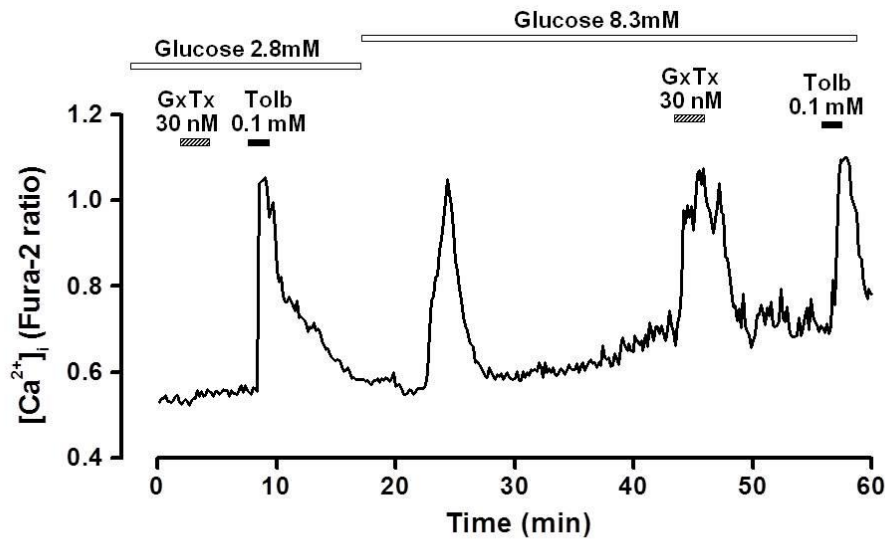


Figure 1. GxTx enhances glucose-induced $[Ca^{2+}]_i$ increases in β -cells of mice. Basal calcium level at 2.8 mM glucose was not altered by administration of GxTx (30 nM). On the other hand, GxTx enhanced glucose-induced $[Ca^{2+}]_i$ increases at 8.3 mM glucose. Tolbutamide (Tolb; 0.1 mM) induced $[Ca^{2+}]_i$ increase both at 2.8 and 8.3 mM glucose concentration. n = 60-74 (single β -cells).

3.1.2 Glucose-induced $[Ca^{2+}]_i$ increases is enhanced in β -cells of Kv2.1^{+/-} mice

Next I examined the effect of genetic blockade of Kv2.1 channel by using Kv2.1^{+/-} mice. Glucose (8.3 mM)-induced $[Ca^{2+}]_i$ increases was enhanced in Kv2.1^{+/-} β -cells (Fig. 2B) compared to wild type β -cells (Fig. 2A). In some cells of Kv2.1^{+/-} β -cells showed calcium oscillation during high glucose concentration (Fig. 2B). Basal $[Ca^{2+}]_i$ in the presence of 2.8 mM glucose was not changed between two groups (Fig. 2C). Area under the curve (AUC) for 30 minutes of 8.3 mM glucose-induced $[Ca^{2+}]_i$ increases was significantly enhanced in Kv2.1^{+/-} β -cells compared to wild type β -cells (Fig. 2D)

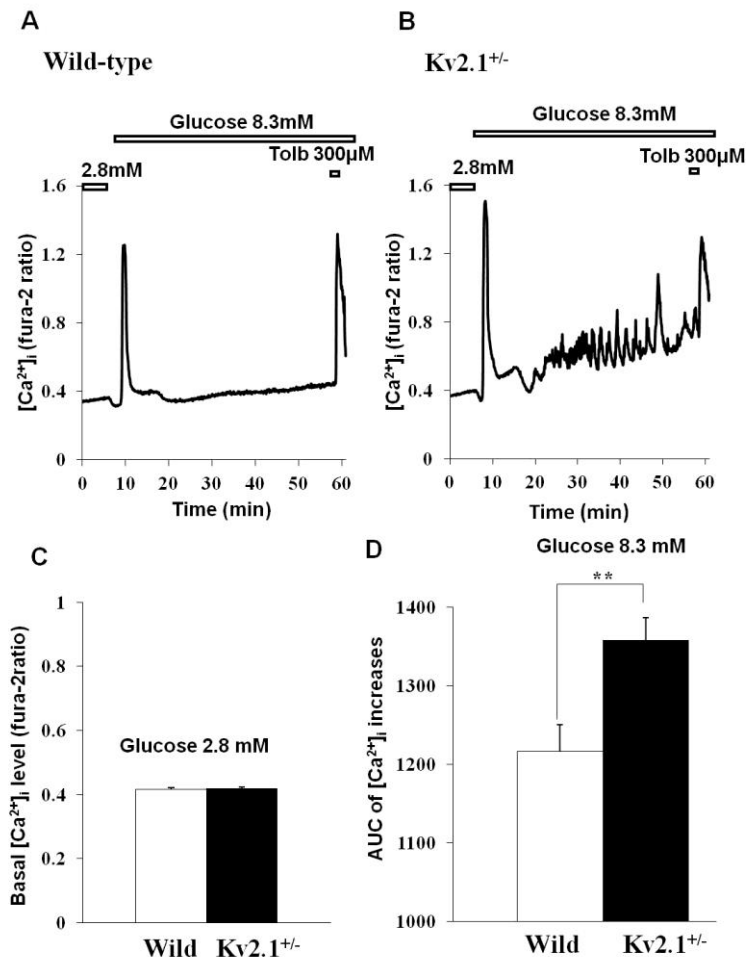


Figure 2. Glucose-induced $[Ca^{2+}]_i$ increases is enhanced in β -cells of Kv2.1^{+/-} mice.
A and B. Representative traces of glucose-induced $[Ca^{2+}]_i$ increases in wild-type β -cells

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and Kv2.1^{+/-} β -cells. **C.** There was no difference on basal calcium level between wild type and Kv2.1^{+/-} β -cells. **D.** The AUC of glucose-induced $[Ca^{2+}]_i$ was significantly higher in β -cells of Kv2.1^{+/-} mice compared to wild-type β -cells. **** $P < 0.01$.** n = 30-68 single β -cells.

3.1.3 GxTx enhances glucose-induced insulin release in pancreatic islets of mice

In order to examine insulin release from isolated islet of pancreatic islets β -cells, groups of 10 islets were incubated in the present of 2.8 mM glucose (basal), 8.3 mM glucose (stimulatory) with or without GxTx (30 nM). At 2.8 mM glucose, administration of GxTx (30 nM) did not affect insulin release in mouse isolated islets. Glucose (8.3 mM) induced insulin release in mouse isolated islets. Furthermore, GxTx (30 nM) enhanced glucose (8.3 mM)-induced insulin release (Fig. 3).

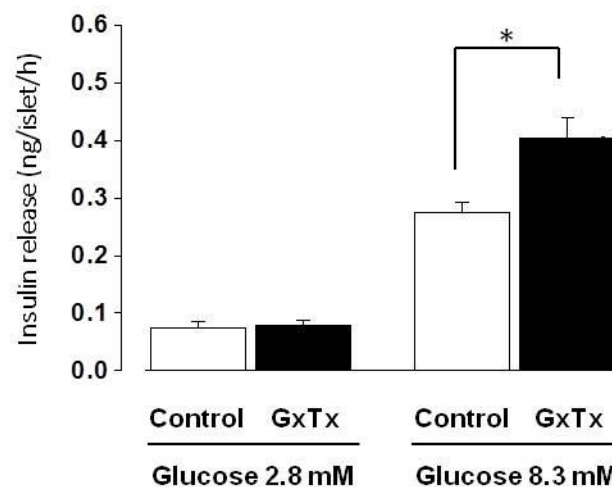


Figure 3. GxTx enhances glucose-induced insulin release in pancreatic islets of mice. There was no difference on basal insulin release between control and administration of GxTx (30 nM). In contrast, at 8.3 mM glucose, GxTx (30 nM) enhanced glucose-induced insulin release compared to control. *** $P < 0.05$** vs control. n = 7-8 (tubes of batch incubation).

3.1.4 Glucose-induced insulin release is enhanced in pancreatic islets of Kv2.1^{+/-} mice

In the presence of 2.8 mM glucose, there was no difference on basal insulin release between islets from Kv2.1^{+/-} mice and wild-type mice. Glucose (8.3 mM)-induced insulin release was significantly enhanced in Kv2.1^{+/-} islets compared to wild-type (Fig. 4).

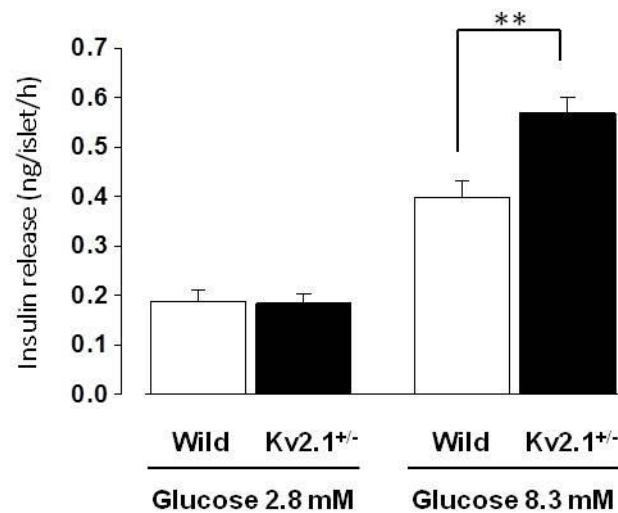


Figure 4. Glucose-induced insulin release is enhanced in pancreatic islets of Kv2.1^{+/-} mice. There was no difference on basal insulin release between islets from Kv2.1^{+/-} mice and wild-type mice. Glucose (8.3 mM)-induced insulin release was significantly enhanced in islets of Kv2.1^{+/-} mice. ** $P < 0.01$ vs wild-type. $n = 7-19$ (tubes of batch incubation).

3.2 Chapter II. Blockade of Kv2.1 channels enhances exendin-4-induced $[Ca^{2+}]_i$ increases and insulin release in pancreatic islets β -cells

3.2.1 β -cell Kv currents are partially blocked by low concentration of GxTx (3 nM) and Kv2.1 hetero-KO

The Kv channel currents in mouse β -cells under conventional whole-cell clamp were measured in the presence of 100 μ M tolbutamide to inhibit the K_{ATP} channel and thereby exclude involvement of this channel in the currents. In the presence of 5.6 mM glucose, a constant depolarizing pulse from -70 to 0 mV every 20 seconds evoked outward Kv currents and the currents decreased by 14 % upon exposure to GxTx (3 nM) (Fig. 5A). At a holding potential of 0 mV, GxTx (3 nM) significantly decreased the current densities to 28.8 ± 2.5 pA/pF from 33.6 ± 3.3 pA/pF ($P < 0.05$, $n = 9$). In β -cells of Kv2.1^{+/-} mice, Kv currents were diminished by 26 % compared to wild-type littermates (Fig. 5B). At a holding potential of 0 mV, the current densities in β -cells of Kv2.1^{+/-} and wild-type mice were 25.2 ± 2.5 pA/pF and 34.2 ± 2.8 pA/pF, respectively ($P < 0.05$, $n = 20$).

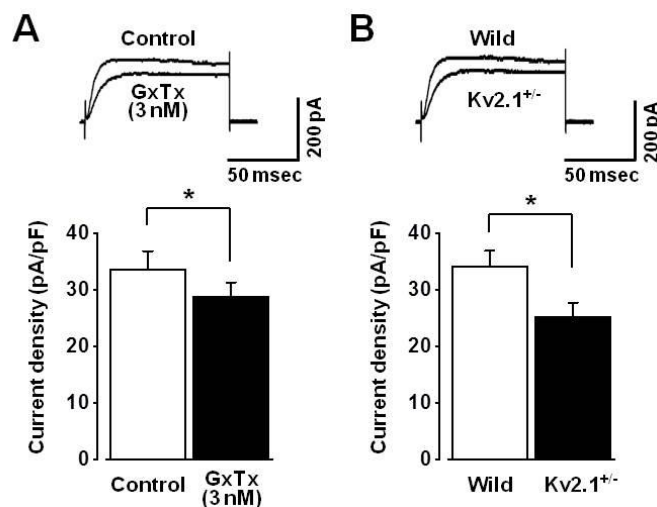


Figure 5. β -cell Kv currents are partially blocked by low concentration of GxTx (3 nM) and Kv2.1 hetero-KO. Current traces evoked by a step pulse to 0 mV from a holding potential of -70 mV were measured in a mouse single β -cell under conventional

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whole-cell clamp mode. Data were recorded in the presence of 5.6 mM glucose and 100 μ M tolbutamide. **A.** Upper panel: Exposure to low concentration of GxTx (3 nM) partially decreased the amplitudes of delayed outward currents. Lower panel: GxTx (3 nM) significantly decreased peak amplitude of the current density in β -cells ($n = 9$ single β -cells). $*P < 0.05$. **B.** Upper panel: Typical traces of Kv currents in β -cells of Kv2.1^{+/-} mice and wild-type littermates. Lower panel: Peak amplitudes of Kv current density were diminished in β -cells of Kv2.1^{+/-} mice compared with wild-type littermates. $*P < 0.05$. $n = 20$ (single β -cells).

3.2.2 GxTx enhances exendin-4-induced $[Ca^{2+}]_i$ increases in β -cells of mice

Effects of GLP-1 analog, Kv2.1 channel blocker, and their combination were examined on β -cell $[Ca^{2+}]_i$, a principal mediator of insulin release. A rise in the glucose concentration from 2.8 to 8.3 mM elicited $[Ca^{2+}]_i$ increases in a biphasic manner, transient large increases followed by sustained moderate increases. During the period with the sustained $[Ca^{2+}]_i$ increases in the presence of 8.3 mM glucose, Ex-4 (30 pM) (Fig. 6A), and GxTx at 3 nM that reduced β -cell Kv currents by 14 % were added (Fig. 6B). These treatments tended to increase $[Ca^{2+}]_i$ in mouse single β -cells. However, the AUC, which corresponds to an integrated $[Ca^{2+}]_i$ increase for 3 min during stimulation, did not show statistically significant difference (Fig. 6F) with either Ex-4 or GxTx. In contrast, when Ex-4 and GxTx at low concentrations at which each agent alone was ineffective were combined, they acted synergistically to evoke marked $[Ca^{2+}]_i$ increases (Fig. 6C) with significant increases in the AUC of $[Ca^{2+}]_i$ (Fig. 6F). Higher concentrations of each drug alone, Exe-4 at 1 nM and GxTx at 100 nM, significantly increased $[Ca^{2+}]_i$ at 8.3 mM glucose (data not shown). Nifedipine (10 μ M), an L-type Ca^{2+} channel blocker, completely inhibited the $[Ca^{2+}]_i$ increase induced by the combination of Ex-4 (30 pM) and GxTx (3 nM), suggesting that this combination enhanced Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Fig. 6D & 6F).

In 2.8 mM glucose, the combination of Ex-4 and GxTx had no effect on $[Ca^{2+}]_i$ in β -cells (Fig. 6E & 6F). Furthermore, long term treatment of cells with Ex-4 (30 pM) and GxTx (3 nM) in combination elicited $[Ca^{2+}]_i$ oscillations in many β -cells at 8.3 mM glucose (Fig. 6G), and the incidence of β -cells with $[Ca^{2+}]_i$ oscillations was significantly increased by the combination treatment (Fig. 6H). The combination of Ex-4 (30 pM) and GxTx (3 nM) did not evoke $[Ca^{2+}]_i$ increases at 8.3 mM glucose in the cells that did not respond to 8.3 mM glucose but responded to tolbutamide (data not shown).

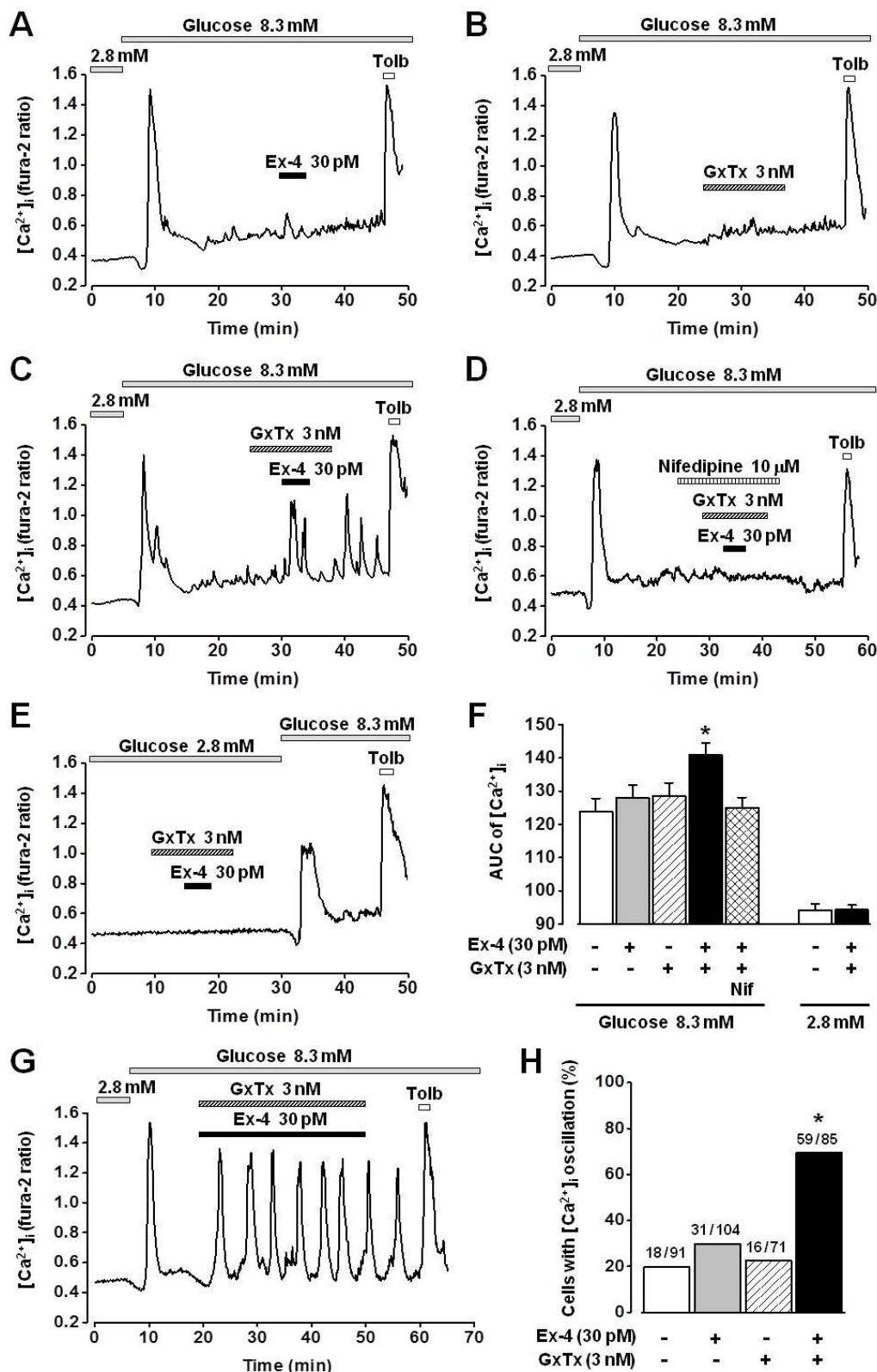


Figure 6. Kv2.1 channel blockade cooperates with exendin-4 to induce $[Ca^{2+}]_i$ increases in mouse β -cells. Representative traces of $[Ca^{2+}]_i$ in single β -cell are expressed by dual-wavelength fura-2 fluorescence ratio (F340/F380). **A and B.** Either

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Ex-4 (30 pM) or GxTx (3 nM) slightly increased $[Ca^{2+}]_i$ in β -cell at 8.3 mM glucose. **C and D.** The substimulatory concentrations of Ex-4 (30 pM) and GxTx (3 nM) in combination at 8.3 mM glucose evoked marked increases in $[Ca^{2+}]_i$ (C), which were completely inhibited by nifedipine (Nif) (10 μ M) (D). **E.** The cooperative effect of Ex-4 (30 pM) and GxTx (3 nM) was not observed at basal 2.8 mM glucose. **F.** AUC of $[Ca^{2+}]_i$ increase was significantly enhanced by combination of Ex-4 and GxTx in a glucose-dependent manner, and this enhancement was completely inhibited by Nif. **G** long-term treatment of cells with Ex-4 (30 pM) and GxTx (3 nM) in combination elicited $[Ca^{2+}]_i$ oscillations in β -cells at 8.3 mM glucose. **H.** Incidence of β -cells with $[Ca^{2+}]_i$ oscillations was increased by the combination treatment. The numbers above each column indicates the number of β -cells with $[Ca^{2+}]_i$ oscillations over that examined. * $P < 0.05$ vs. 8.3 mM glucose. $n = 60-104$ (single β -cells).

3.2.3 Exendin-4-induced $[Ca^{2+}]_i$ increases is augmented in β -cells from Kv2.1^{+/-} mice

To support the potentiation of $[Ca^{2+}]_i$ increase by combined treatment with Kv2.1 channel blocker and GLP-1 agonist, I next examined effects of Ex-4 on the β -cell $[Ca^{2+}]_i$ from Kv2.1^{+/-} mice in which the gene coding Kv2.1 is partially deleted and β -cell Kv currents were reduced by 26 %. Glucose (8.3 mM)-induced $[Ca^{2+}]_i$ increase was moderately enhanced in Kv2.1^{+/-} β -cells compared to those in wild-type β -cells (Fig. 7D), while basal $[Ca^{2+}]_i$ at 2.8 mM glucose was identical (Fig. 7C). In the presence of 8.3 mM glucose, Ex-4 (30 pM) only slightly increased $[Ca^{2+}]_i$ in wild-type β -cells (Fig. 7A), but evoked large $[Ca^{2+}]_i$ increases in Kv2.1^{+/-} β -cells (Fig. 7B). The AUC of Ex-4-induced $[Ca^{2+}]_i$ increases was significantly augmented in Kv2.1^{+/-}, compared to wild-type, β -cells (Fig. 7E).

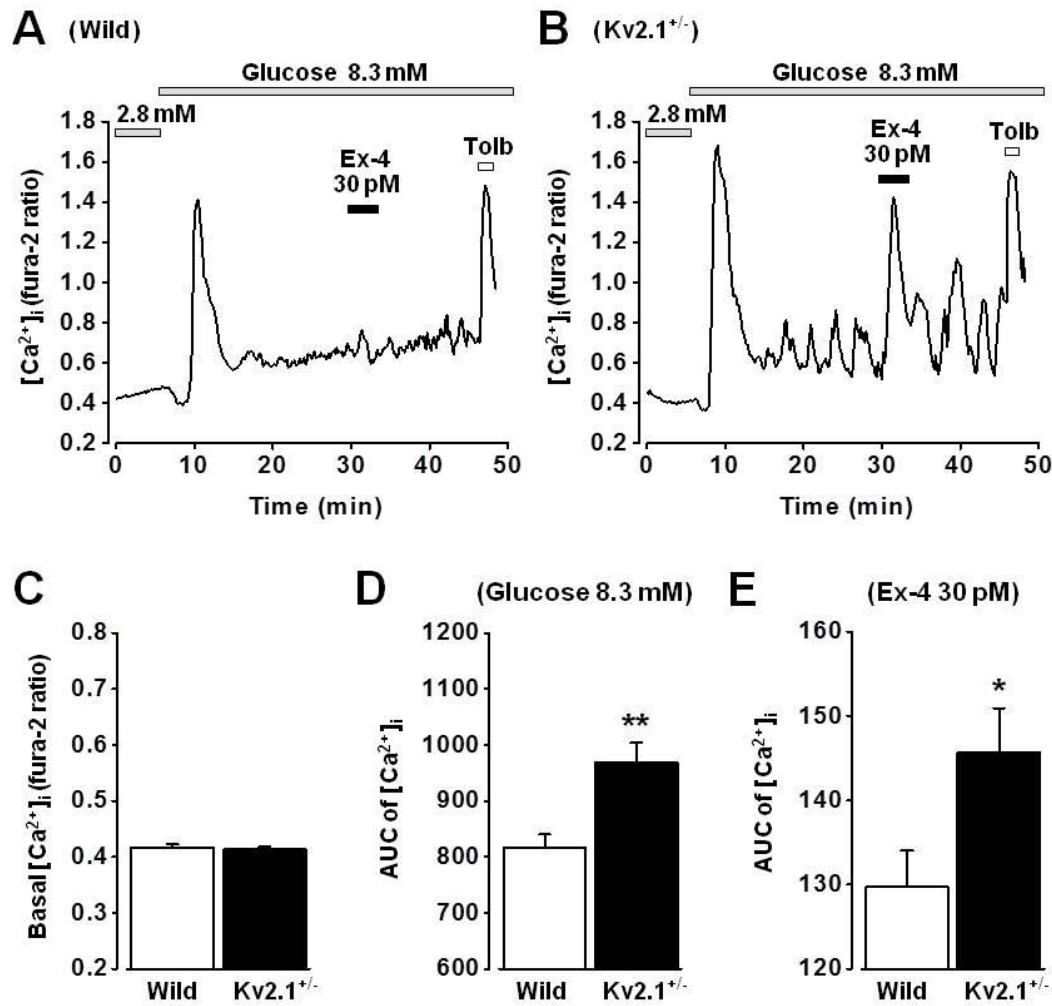


Figure 7. Exendin-4-induced $[Ca^{2+}]_i$ increases are augmented in β -cells from Kv2.1^{+/-}. *A and B.* At 8.3 mM glucose, low concentration of Ex-4 (30 pM) evoked large increase in $[Ca^{2+}]_i$ in Kv2.1^{+/-} β -cells (B), in contrast to slight increase in $[Ca^{2+}]_i$ in wild-type β -cells (A). *C and D.* AUC of Ex-4-induced $[Ca^{2+}]_i$ increases for 20 minutes in Kv2.1^{+/-} β -cells was significantly greater than that in wild-type β -cells (D) while basal $[Ca^{2+}]_i$ levels at 2.8 mM glucose was identical (C). *E.* AUC of Ex-4-induced $[Ca^{2+}]_i$ increases in Kv2.1^{+/-} β -cells. * $P < 0.05$, ** $P < 0.01$ vs. wild-type. $n = 55-60$ (single β -cells).

3.2.4 GxTx and exendin-4 cooperatively increase $[Ca^{2+}]_i$ in a protein kinase-A-dependent manner in β -cells of wild type mice

Activation of GLP-1 receptor is linked to cAMP signaling, lead to activation of protein kinase-A (PKA) and exchange proteins directly activated by cAMP (Epac).

Synergistic action of Ex-4 and GxTx to increase $[Ca^{2+}]_i$ was attenuated by pretreatment with a PKA inhibitor H-89 (10 μ M) (Fig. 8A and 8B) with significant reduction of the AUC of $[Ca^{2+}]_i$ increases (Fig. 8C). Furthermore, a low concentration of 6-Phe-cAMP (Phe-cAMP) (3 μ M), a PKA activator, only slightly increased $[Ca^{2+}]_i$ in β -cells (Fig. 8D). In the presence of GxTx (3 nM), however, this PKA activator induced marked increases in $[Ca^{2+}]_i$ (Fig. 8E & 8F), showing a synergistic effect of PKA activator and GxTx. In contrast, synergistic effect was not observed between GxTx and an Epac activator 8-pCPT-2'-O-Me-cAMP-AM (CPT-cAMP) (3 μ M) (Fig. 8E & 8F). These results suggest that blockade of Kv2.1 channels may interact with GLP-1 receptor-operated PKA signaling route, thereby inducing $[Ca^{2+}]_i$ increases in β -cells.

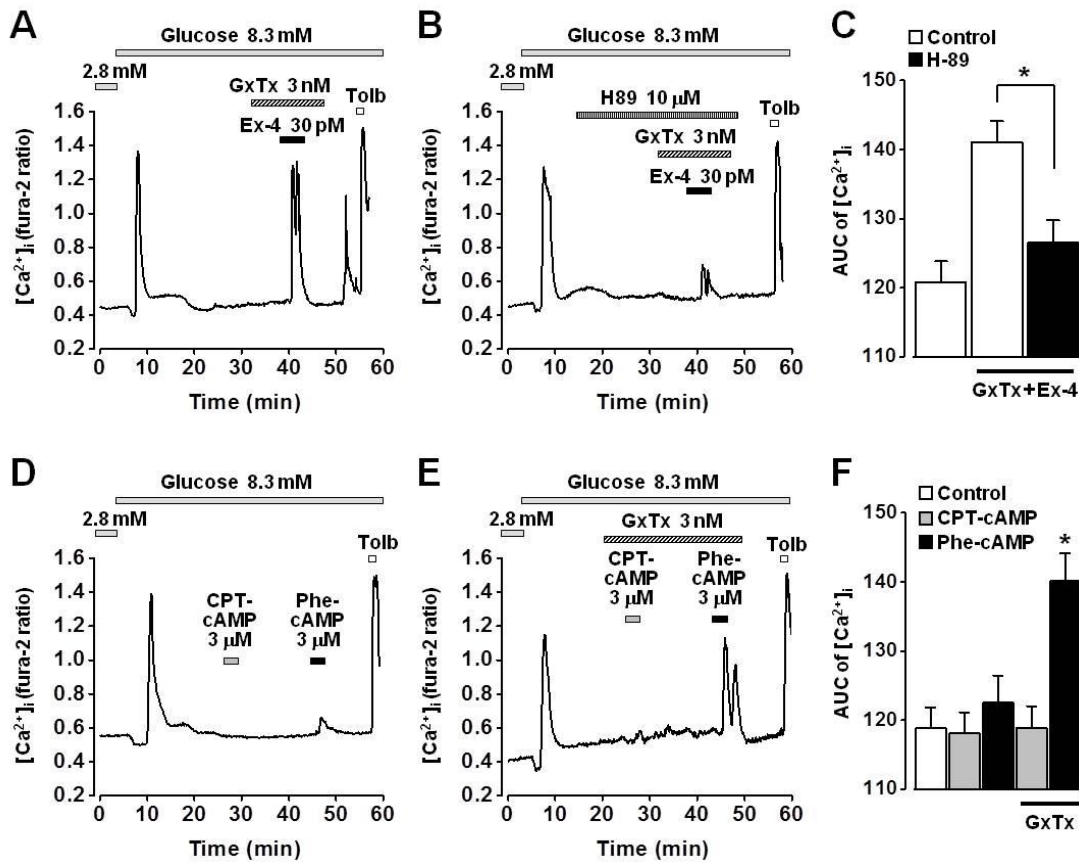


Figure 8. Kv2.1 channel blockade and exendin-4 cooperatively increase $[Ca^{2+}]_i$ in a protein kinase-A-dependent manner in β -cells. A and B. Synergistic action of Ex-4 and GxTx to increase $[Ca^{2+}]_i$ at 8.3 mM glucose (A) was attenuated by pretreatment

(Legend to Figure 8 continued to next page)

(Continued Legend to Figure 8)

with a PKA inhibitor H-89 (10 μ M) (B). **C.** AUC of $[Ca^{2+}]_i$ increases by Ex-4 and GxTx was significantly inhibited by H-89. **D.** PKA activator, 6-Phe-cAMP (Phe-cAMP) (3 μ M), slightly increased $[Ca^{2+}]_i$ and Epac activator, 8-pCPT-2'-O-Me-cAMP-AM (CPT-cAMP) (3 μ M), little increased $[Ca^{2+}]_i$ in β -cells. **E.** In the presence of GxTx (3 nM), the PKA activator evoked marked increases in $[Ca^{2+}]_i$ while Epac activator had little effect on $[Ca^{2+}]_i$ in β -cells. **F.** AUC of $[Ca^{2+}]_i$ increases was significantly elevated by Phe-cAMP with GxTx but not by CPT-cAMP with GxTx. * $P < 0.05$ vs. control of 8.3 mM glucose. n = 48-56 (single β -cells).

3.2.5 GxTx enhances exendin-4-induced insulin release in pancreatic islets of mice

Effects of Ex-4 and GxTx on the insulin release in mouse isolated islets were measured under basal (2.8 mM) and stimulatory (8.3 mM) glucose conditions. Insulin release from islets under batch-incubation conditions was larger with 8.3 mM glucose than 2.8 mM glucose (Fig. 9A). The glucose-induced insulin release tended to be enhanced by low concentration of either Ex-4 (30 pM) or GxTx (3 nM), but not statistically significant (Fig. 9A). Combination of Ex-4 (30 pM) and GxTx (3 nM) significantly enhanced the glucose-induced insulin release, without affecting insulin release at 2.8 mM glucose (Fig. 9A). Furthermore, GxTx (3 nM) enhanced insulinotropic action of Ex-4 at 30-100 pM, while it did not affect the effect of Ex-4 at higher concentration of 1-10 nM, showing left-ward shift of the concentration-response curve of Ex-4 with EC_{50} values of 127.6 pM for control and 34.1 pM for GxTx, respectively (Fig. 9B). These results indicate that GxTx potentiates the Ex-4 action to promote glucose-induced insulin release. GxTx at a higher concentration of 1 μ M by itself markedly enhanced 8.3 mM glucose-induced insulin release, and Ex-4 (30 pM) failed to further potentiate it (Fig. 9A), suggesting that Ex-4 (30 pM) partially inhibits Kv2.1 channels as one of its action mechanisms.

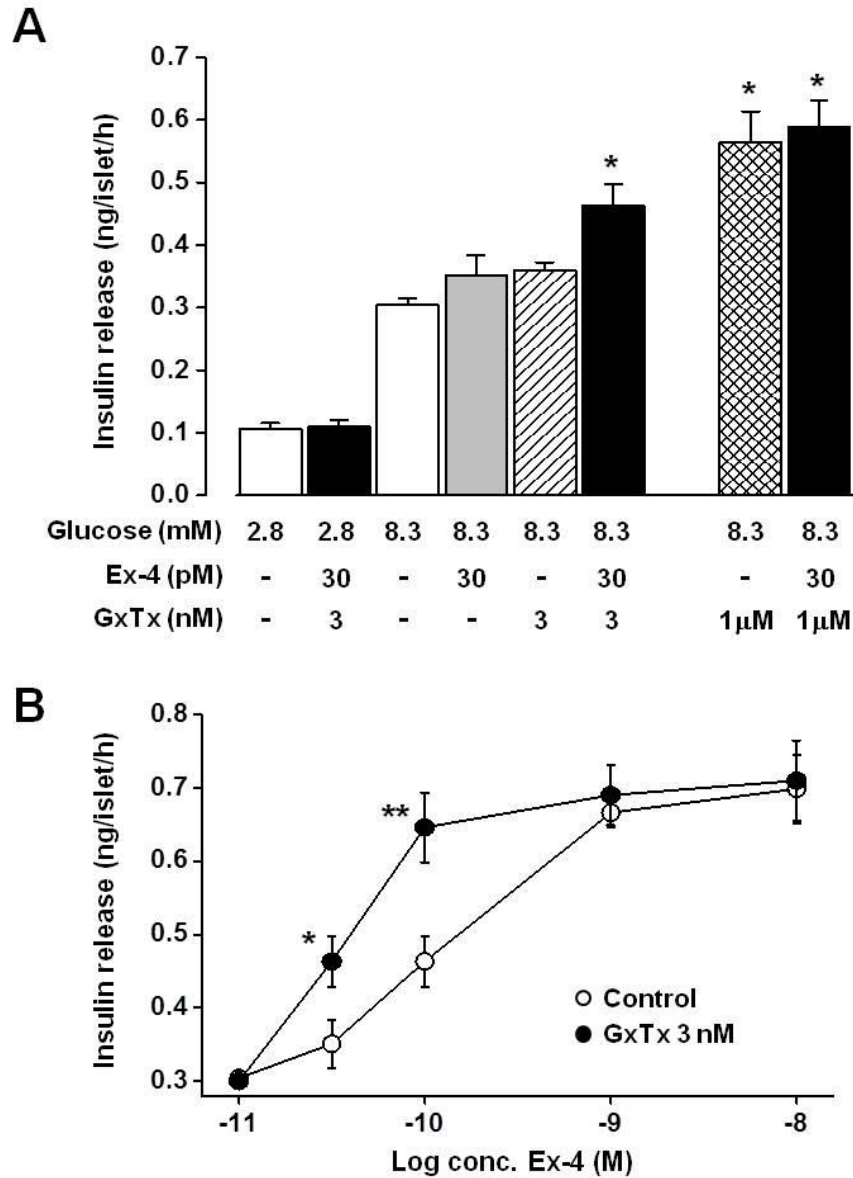


Figure 9. Kv2.1 channel blockade cooperates with exendin-4 to enhance insulin release in mouse isolated islets. A. In isolated islets from wild-type mice, glucose (8.3 mM)-induced insulin release was significantly enhanced by combination of Ex-4 (30 pM) and GxTx (3 nM), either of which individually failed to significantly increase the insulin release. High concentration of GxTx at 1 μ M alone enhanced insulin at 8.3 mM glucose, and Ex-4 (30 pM) failed to potentiate it. **B.** GxTx (3 nM) left-ward shifted concentration-response curve of Ex-4 with EC50 values of 127.6 pM for control and 34.1 pM for GxTx, respectively. * $P < 0.05$, ** $P < 0.01$ vs. control (n = 8-9 tubes of islets from six mice for batch incubation).

3.2.6 Exendin-4-induced insulin release is enhanced in pancreatic islets of Kv2.1^{+/-} mice

In isolated islets from Kv2.1^{+/-} mice, whose Kv currents in β -cells were reduced by 26 %, glucose (8.3 mM)-induced insulin release was significantly greater than that from wild-type littermate (Fig. 10), while basal levels of insulin release at 2.8 mM glucose were not altered. The glucose (8.3 mM)-induced insulin release in Kv2.1^{+/-} islets was further elevated by low concentration of Ex-4 (30 pM), which did not significantly altered glucose-induced insulin release in wild-type islets (Fig. 10), supporting potentiation of Ex-4 effects by blockade of Kv2.1 channels. Basal insulin release at 2.8 mM glucose was not affected by Ex-4 (30 pM) in wild-type and Kv2.1^{+/-} islets (Fig.10). In Kv2.1^{-/-} islets, addition of GxTx at 30 nM failed to further increase the glucose-induced insulin release (data not shown), confirming that GxTx promoted insulin release primarily via suppressing Kv2.1 channels (27).

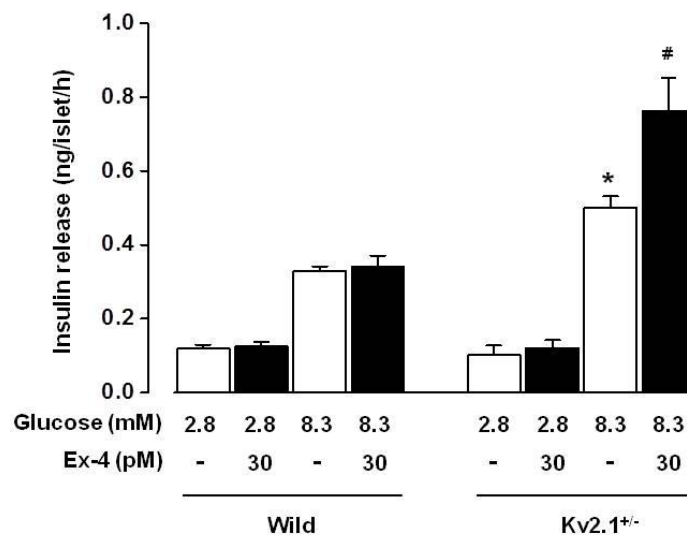


Figure 10. Exendin-4-induced insulin release is enhanced in isolated islets from Kv2.1^{+/-} mice. In isolated islets from Kv2.1^{+/-} mice, the glucose (8.3 mM)-induced insulin release was enhanced by the sub-threshold concentration of Ex-4 (30 pM). **P* < 0.05 vs. 8.3 mM glucose in wild-type; #*P* < 0.05 vs. 8.3 mM glucose in Kv2.1^{+/-}. n = 8-10 (tubes of batch incubation).

3.3 Chapter III. Genetic and pharmacologic blockade of Kv2.1 channel and GLP-1 agonist cooperatively improve glucose tolerance and increase plasma insulin

3.3.1 Genetic blockade of Kv2.1 channel and GLP-1 agonist cooperatively improve glucose tolerance and increase plasma insulin level

To assess the effect of Kv2.1 blockade and GLP-1 agonist in combination *in vivo*, I examined the effects of liraglutide, a long-acting GLP-1 agonist, in Kv2.1^{+/-} mice. In Kv2.1^{+/-} mice following overnight fasting, increases in blood glucose levels at 15-120 minutes and plasma insulin responses at 15 and 30 minutes of IPGTT were not significantly different from those of wild-type mice (Fig. 11A & 11B), although insulin responses at 15 and 30 minutes tended to be slightly enhanced. In wild-type mice, administration of this low dose of liraglutide (3 nmol/kg i.p.) failed to significantly alter blood glucose and plasma insulin levels during IPGTT (Fig. 11A & 11B). In contrast, administration of this low dose of liraglutide in Kv2.1^{+/-} mice significantly suppressed blood glucose increases at 60 and 120 minutes and enhanced insulin responses at 15 minutes during IPGTT (Fig. 11A & 11B). In OGTT, furthermore, Kv2.1^{+/-} mice exhibited improved glucose tolerance at 60 minutes (Fig. 11C) and increased plasma insulin levels at 15 minutes after glucose challenge (Fig. 11D), compared to wild-type mice. This result indicates that Kv2.1 channel regulates the insulintropic actions of endogenous GLP-1 and possibly other incretin hormones released by an oral glucose challenge.

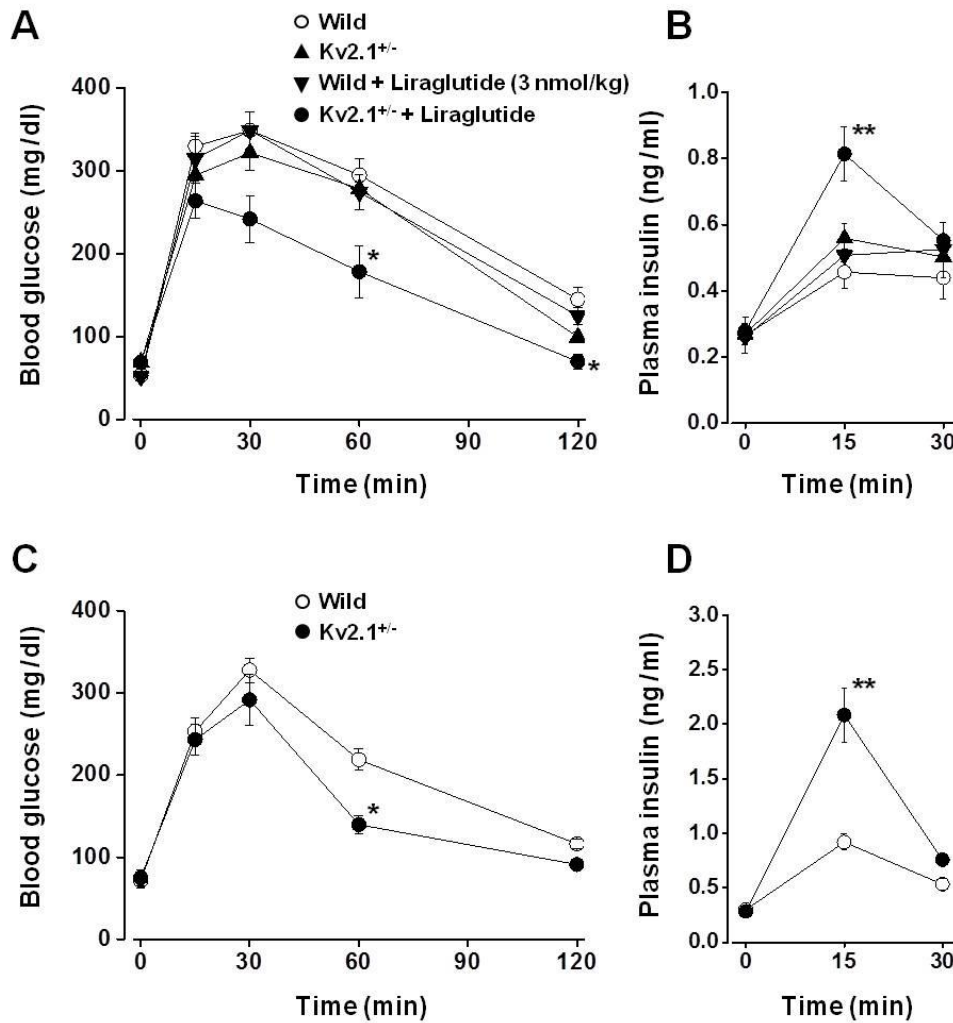


Figure 11. Genetic blockade of Kv2.1 channel and GLP-1 agonist cooperatively improve glucose tolerance and increase plasma insulin level. *A.B.* In IPGTT, following overnight fasting, 2 g/kg glucose was i.p. injected into wild type and Kv2.1^{+/-} mice, while test agents were administered 30 minutes before glucose injection. In Kv2.1^{+/-} mice, low dose (3 nmol/kg, i.p.) of liraglutide, which was ineffective in wild type mice, significantly suppressed blood glucose increases at 60 and 120 minutes and enhanced insulin responses at 15 minutes of IPGTT. * $P < 0.05$, ** $P < 0.01$ vs. control. $n = 8-10$. *C and D.* In OGTT, following overnight fasting, 2 g/kg glucose was orally injected into mice. Kv2.1^{+/-} mice exhibited improved glucose tolerance at 60 minutes and increased plasma insulin levels at 15 minutes after glucose challenge (D), compared to wild-type mice. * $P < 0.05$, ** $P < 0.01$ vs. wild-type. $n = 7$.

3.3.2 Pharmacological blockade of Kv2.1 channel and GLP-1 agonist in combination improve glucose tolerance and increase plasma insulin level

I next conducted an IPGTT using GxTx and liraglutide in type 2 diabetic db/db mice and NSY mice. Following overnight fasting, either GxTx or liraglutide, or their combination, all at sub-threshold doses, was administered to db/db mice aged 7 weeks or NSY mice aged 20 weeks 30 minutes before glucose challenge. A single administration of either GxTx (100 nmol/kg i.p.) or liraglutide (3 nmol/kg i.p.) did not significantly suppress blood glucose increases in IPGTT. In contrast, combination of GxTx and liraglutide significantly suppressed blood glucose increases in both db/db mice (Fig. 12A) and NSY mice (Fig. 12C). During IPGTT, plasma insulin levels exhibited no significant increase in response to either GxTx or liraglutide. GxTx and liraglutide in combination, however, exhibited a magnificent increases in plasma insulin levels at 15 and 30 min after glucose challenge, compared to the saline-injected control db/db (Fig. 12B) and NSY mice groups (Fig.12D).

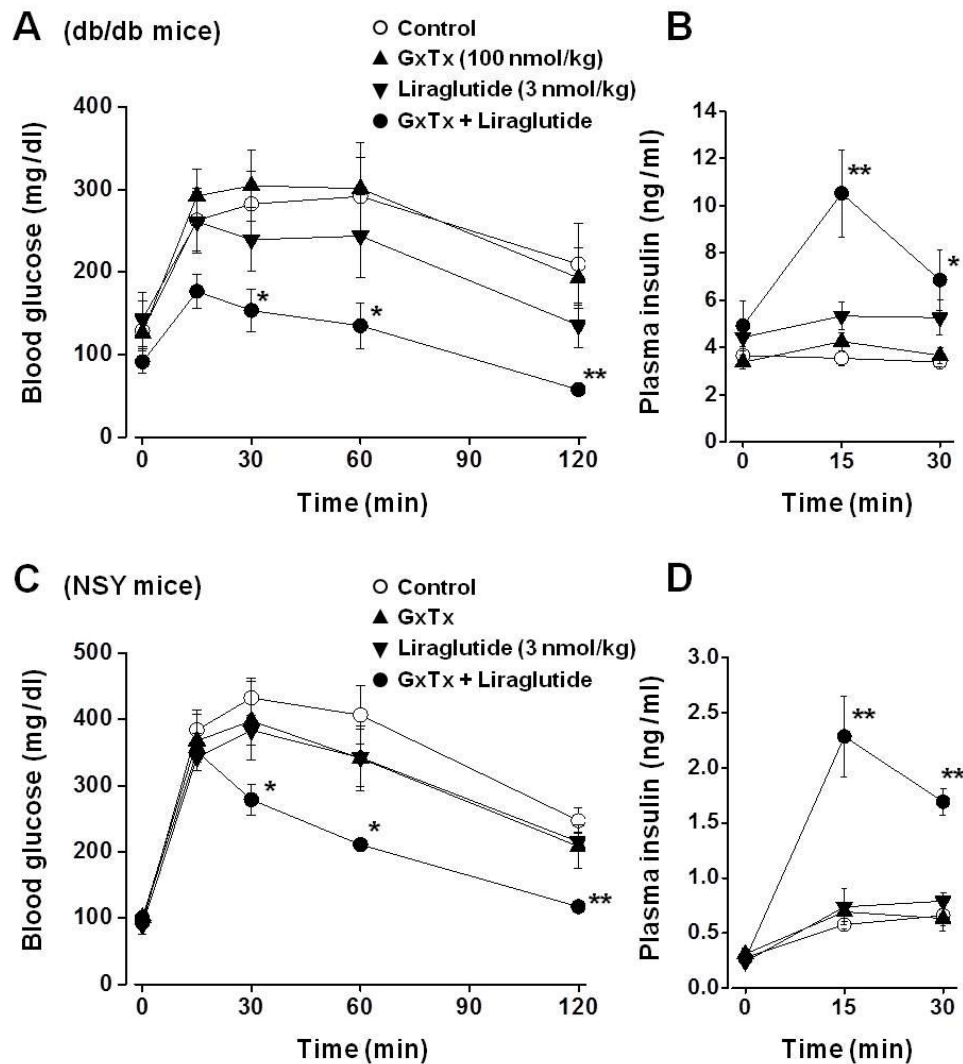


Figure 12. GxTx and liraglutide in combination improve glucose tolerance and increase plasma insulin in type 2 diabetic model mice. In IPGTT, following overnight fasting, 1 g/kg glucose was i.p. injected into db/db mice and 2 g/kg glucose to NSY mice, and test agents were administered 30 min before glucose injection. **A and B.** In db/db mice, GxTx (100 nmol/kg, i.p.) and liraglutide (3 nmol/kg, i.p.) individually failed to significantly alter blood glucose (A) and plasma insulin levels (B), but when combined, they significantly improved glucose tolerance at 30, 60 and 120 min (A) and increased plasma insulin levels at 15 and 30 min after glucose challenge (B), compared to saline-injected control group. **C and D.** In NSY mice, GxTx (100 nmol/kg, i.p.) and liraglutide (3 nmol/kg, i.p.) individually failed to significantly alter blood glucose (C) and plasma insulin levels (D). When combined, they significantly improved glucose tolerance at 30, 60 and 120 min (C) and increased plasma insulin at 15 and 30 min (D), compared to saline-injected control. * $P < 0.05$, ** $P < 0.01$ vs. control. n = 10.

3.4 Chapter IV. Combined treatment with GLP-1 agonist and Kv2.1 channel blocker had negligible effect on systolic blood pressure and heart rate in db/db mice

To assess potential effects of GxTx and GLP-1 agonist on the cardiovascular system, blood pressure and heart rate were measured before and 30 minutes after injection of GxTx or GxTx+liraglutide in db/db mice. Neither GxTx (100 nmol/kg, i.p.) nor combination of GxTx (100 nmol/kg, i.p.) and liraglutide (3 nmol/kg, i.p.) affected systolic blood pressure (96.42 ± 8.12 mmHg and 101.83 ± 8.11 mmHg, respectively, $n = 4$) and heart rate (472.25 ± 14.93 bpm and 472.75 ± 27.25 bpm, respectively, $n = 4$), indicating that the GxTx and liraglutide combined at these doses are without effect on blood pressure and heart rate while they are strikingly potent in inducing insulin release.

4 DISCUSSION

The present study demonstrated that blockade of Kv2.1 channels enhances the glucose-induced $[Ca^{2+}]_i$ increases and insulin release in pancreatic islets β -cells. These results suggest that Kv2.1 channel physiologically restrict glucose-induced Ca^{2+} influx and thereby attenuate insulin secretion in β -cells. GLP-1 receptor agonist Ex-4 and Kv2.1 channel blocker GxTx at sub-threshold concentrations, when combined, markedly increased insulin release in islets and evoked $[Ca^{2+}]_i$ increases in β -cells in a glucose-dependent manner. These results indicate that GLP-1 agonist and Kv2.1 channel blocker act synergistically to activate β -cells and induce insulin secretion. Moreover, Kv2.1 channel blockade and GLP-1 agonist cooperate to increase plasma insulin and improve glucose tolerance *in vivo*. In Kv2.1^{+/-} mice, a low dose of liraglutide, which was ineffective in wild-type mice, significantly enhanced insulin release and suppressed blood glucose elevation in IPGTT. Sub-threshold doses of GxTx and GLP-1 receptor agonist liraglutide in combination markedly increased plasma insulin and improved glucose tolerance in diabetic db/db mice and NSY mice. These results indicate that Kv2.1 channels physiologically limit insulintropic actions of endogenous GLP-1 and possibly other incretin hormones, and that blockade of Kv2.1 channels provides a novel tool to enhance the insulintropic potency of GLP-1-based drugs.

It has been reported that Kv2.1 channels are involved in repolarization of pancreatic β -cells and thereby limit insulin release (9,10). The present study examined the physiological role of Kv2.1 channel by using pharmacological and genetic blockade of Kv2.1 channel. The results showed that either pharmacological blockade of Kv2.1 channel by using GxTx or genetic blockade of Kv2.1 in Kv2.1^{+/-} mice, enhances the glucose-induced $[Ca^{2+}]_i$ increases and insulin release in pancreatic islet β -cells. These

results confirmed previous studies that this channel physiologically limits glucose-induced insulin release in pancreatic β -cells (6,9).

GLP-1 agonists have been shown to improve glucose intolerance in type 2 diabetic patients (28) with a lower risk of hypoglycemia than sulfonylureas. It has been shown, however, that GLP-1 agonists often causes adverse events such as nausea and vomiting, and that their incidence is dose-dependent (16,17). The present study showed that the use of Kv2.1 channel blocker, even at sub-threshold concentrations, markedly reduces the dose of GLP-1 agonists required for their insulinotropic and blood glucose-lowering effects. This finding opens a new clinical avenue to reduce the dose and associated adverse effects of GLP-1 agonists, while achieving the same glycemic control in type 2 diabetes. It has been reported that both plasma GLP-1 level and insulinotropic GLP-1 action are reduced in type 2 diabetic patients, contributing to impaired insulin release and hyperglycemia (29-31). In these cases, DPP-4 inhibitors could restore the plasma GLP-1 level and Kv2.1 channel blocker could restore the GLP-1 action, and these two processes could collaborate to augment the eventual insulinotropic GLP-1 effect in islet β -cells. Thus, the clinically applicable Kv2.1 channel blocker, if available in the future, could amplify the potency of DPP-4 inhibitors. I here propose a novel strategy to intensify the therapy of GLP-1 agonists and DPP-4 inhibitors by using Kv2.1 channel blockers. In addition, DPP-4 inhibitors are known to elevate circulating levels of not only GLP-1 but also other incretin hormones, which include glucose-dependent insulinotropic polypeptide (GIP) and pituitary adenylate cyclase-activating polypeptide (32,33) that act through cAMP signaling in islet β -cells. The insulinotropic effects of these hormones could also be enhanced by blockade of Kv2.1 channels. This possibility remains to be studied.

The Kv2.1 channel is expressed not only in islets but in other tissues including cardiovascular system (2), suggesting that the use of Kv2.1 channel blockers possibly induces side effects. In the present study, however, the effect of GLP-1 agonists was enhanced by Kv2.1 channel blockers at the dose in which this drug alone was ineffective on islets and β -cells. Furthermore, Kv2.1 channel blocker at the dose used in this study had no effect on the blood pressure. Therefore, when combined with GLP-1 agonists, the Kv2.1 channel blocker at relatively low doses might preferentially act on islets without significant adverse effects.

The effects of GLP-1 are mediated by stimulation of adenylate cyclase and subsequent increase in intracellular cyclic AMP, which lead to activation of PKA and/or Epac pathways (34-36). Present study indicates that synergistic effect of sub-stimulatory concentrations of GxTx and Ex-4 to increase $[Ca^{2+}]_i$ in β -cells was attenuated by pretreatment with a PKA inhibitor H-89. Furthermore, when combined with GxTx, a sub-stimulatory concentration of PKA activator Phe-cAMP (3 μ M), but not Epac activator CPT-cAMP (3 μ M), evoked $[Ca^{2+}]_i$ increases in β -cells. These results suggest that the Kv2.1 channel blockade interacts with the PKA, but not Epac, branch of cAMP signaling in β -cells. Additionally, it is possible that the relatively low concentrations of GLP-1 agonists used in this study produces cAMP to a marginal level incapable of activating Epac, since higher concentrations of cAMP are required for activating Epac pathway (37,38). However, the data cannot exclude an additional involvement of Epac signaling, since interplay of PKA and Epac was suggested to be implicated in cAMP-dependent stimulation of $[Ca^{2+}]_i$ increases in β -cells and insulin secretion (39).

Regarding the further mechanism for interaction between PKA and Kv channel, we have previously reported that a PKA activator Phe-cAMP attenuates β -cell Kv

currents (40). Consistent with our observations, the activation of GLP-1 receptor phosphorylates Kv2.1 in β -cells via PKA (41) and reduces Kv currents to antagonize β -cell repolarization in a cAMP/PKA-dependent manner (20,42). Under these conditions, additional treatment of cells with sub-stimulatory concentration of GxTx may further inhibit the Kv2.1 channels and induce a greater depolarization, leading to enhanced Ca^{2+} influx and insulin secretory response. As an additional or alternative target process in β -cells, cAMP signal-mediated promotion of insulin exocytosis (18) and/or facilitation of L-type Ca^{2+} channel activities (43) could be involved, which remains to be established. On the other hand, we have reported that TRPM2, a non-selective cation channel, mediates GLP-1 signal to promote insulin release (44,45). Hence, it is possible that activation of TRPM2 channels by GLP-1 could cooperate with blockade of Kv2.1 channels, resulting in effective depolarization of β -cells. This potential implication of TRPM2 channels in the synergistic effects of GLP-1 agonist and GxTx remains to be determined.

In conclusion, Kv2.1 channels physiologically limit glucose-induced Ca^{2+} influx and thereby attenuate insulin secretion in pancreatic β -cells. Combination of low doses of GLP-1 agonist and Kv2.1 channel blocker exert strong *in vivo* insulintropic and blood glucose-lowering effects in type 2 diabetic mice. Blockade of Kv2.1 channels enhances potency of GLP-1-based drugs, which can lower their dose required to improve glucose tolerance in type 2 diabetes and thereby reduce the frequency of their adverse effects. This study provides a novel avenue to make the GLP-1-based diabetes therapy more effective and reliable.

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6 APPENDIX

6.1 List of abbreviations

[Ca²⁺]_i	-	Cytosolic Ca ²⁺ concentration
AUC	-	Area under the curve
bpm	-	beat per minute
BSA	-	Bovine serum albumin
cAMP	-	cyclic adenosine 3',5'-monophosphate
DPP-4	-	Dipeptidyl peptidase-4
Epac	-	Exchange proteins directly activated by cAMP
GIP	-	Glucose-dependent insulinotropic polypeptide
GLP-1	-	Glucagon-like peptide-1
GTT	-	Glucose tolerance tests
GxTx	-	Guangxitoxin-1E
HKRB	-	HEPES-added Krebs-Ringer bicarbonate buffer
IPGTT	-	Intraperitoneal glucose tolerance tests
Kv	-	Voltage-gated potassium channels
OGTT	-	Oral glucose tolerance tests
PACAP	-	Pituitary adenylate cyclase-activating polypeptide
PCR	-	Polymerase chain reaction
PKA	-	Protein kinase-A
TRPM2	-	Transient receptor potential melastatin 2

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