Involvement of cAMP-EPAC-TRPM2 activation in glucose- and incretin-induced insulin secretion.

Short running title: TRPM2 signal via cAMP-EPAC in insulin secretion

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Abstract

In pancreatic β-cells, closure of the ATP-sensitive K⁺ (K-ATP) channel is an initial process triggering glucose-stimulated insulin secretion. In addition, constitutive opening of background nonselective-cation channels (NSCCs) is essentially required to effectively evoke depolarization as a consequence of K-ATP channel closure. Thus, it is hypothesized that further opening of NSCC facilitates membrane excitability. We identified a class of NSCC that was activated by exendin-4, GLP-1 and its analogue liraglutide at picomolar levels. This NSCC was also activated by increasing the glucose concentration. NSCC activation by glucose and GLP-1 was a consequence of the activated cAMP/EPAC-mediated pathway, and was attenuated in TRPM2-deficient mice. The NSCC was not activated by PKA activators and was activated by exendin-4 in the presence of PKA inhibitors. These results suggest that glucose- and incretin-activated NSCC (TRPM2) works in concert with closure of the K-ATP channel to effectively induce membrane depolarization to initiate insulin secretion. The present study reveals a new mechanism for regulating electrical excitability in β-cells and for mediating the action of glucose and incretin to evoke insulin secretion, thereby providing an innovative target for the treatment of type 2 diabetes.
Introduction

It has been long proposed that glucose-stimulated insulin secretion in pancreatic β-cells is initiated by closure of the ATP-sensitive K⁺ (K-ATP) channel, followed by membrane depolarization (1). In theory, closure of the K-ATP channel is an important process, but is not sufficient to induce the shift of the membrane potential towards a threshold level, as membrane potential is determined by the overall balance of outward and inward currents. Modest constitutive opening of background inward current through nonselective cation channels (NSCCs) is crucial to facilitate depolarization following K-ATP channel closure (2). This idea suggests that further regulated opening of a class of NSCC may bring about greater depolarization. However, whether glucose metabolism regulates NSCC activity remains unclear.

The incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulino tropic polypeptide (GIP), potentiate insulin secretion in association with increased intracellular Ca²⁺ concentrations at insulin-secreting glucose concentrations (3-5). GLP-1 fails to increase insulin secretion from the islets of mice deficient in TRPM2 (transmembrane receptor potential melastatin 2)(6; 7), a type of NSCC, suggesting that the TRPM2 channel is essential for GLP-1-induced potentiation of glucose-stimulated insulin secretion (8). GLP-1 depolarizes the plasma membrane by
the opening of NSCC in β-cells (2). Several types of NSCC (TRPs) are expressed in insulin-secreting cells (9). The aims of the present study are to determine; (a) the type of NSCC activation (through TRPM2 or other TRPs) that is crucial for signaling after stimulation of the incretin receptor; (b) whether the NSCC is modulated by glucose metabolism; and (c) the underlying mechanism involved in these physiologically and clinically fundamental stimuli.
**Research design and Methods**

Male Wistar rats and C57BL/6J mice (CLEA Japan, Inc., Tokyo) were housed in accordance with the institutional guidelines for animal care in an air-conditioned room with a 12-hour light/dark cycle, and food and water were available ad libitum. The study protocol was approved by the institutional animal ethics committee. Islets of Langerhans were isolated by collagenase digestion from male Wistar rats (age, 8-12 weeks; weight, approximately 200 g), C57BL/6J mice and TRPM2-deficient mice (8 weeks old, 20 g) using a previously reported method (10; 11). Briefly, animals were anesthetized by intraperitoneal injection of pentobarbital (25-100 mg/kg) followed by injection of collagenase (Sigma-Aldrich, Tokyo, Japan; 1.05 mg/ml, dissolved in a 5 mM CaCl$_2$ solution containing HEPES-added Krebs-Ringer bicarbonate buffer (HKRB) [129 mM NaCl, 5 mM NaHCO$_3$, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 2 mM CaCl$_2$, 1.2 mM MgSO$_4$, and 10 mM HEPES, at pH 7.4 with NaOH] directly into the common bile duct. The pancreas was incubated at 37°C for 15 min in HKRB buffer. Collected islets and subsequently dispersed cells were used for insulin release and for electrophysiological experiments, respectively.

TRPM2 knockout mice were kindly provided by Dr. Y. Mori (Kyoto University) (12).

TRPM2-KO mice were backcrossed with the C57B6/J strain for at least nine
generations.

**Measurement of insulin secretion**

Each batch of 10 islets was incubated for 30 min at 37°C in HKRB with 2.8 mM glucose for stabilization, followed by incubation for 5, 15 or 60 min in HKRB with 2.8 mM or 16.6 mM glucose, with and without $10^{-10}$ or $10^{-9}$ M ex-4 or 10 µM 2-APB. Secreted insulin concentrations in supernatants of each test batch were determined with ELISA kits (Morinaga Institute of Biological Science, Yokohama, Japan).

**Measurement of cytoplasmic Ca$^{2+}$ concentration**

Single β-cells were isolated from male C57BL/6J mice and plated on coverslips. Cytoplasmic Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) in β-cells was measured as reported previously (10). Briefly, β-cells were superfused with HKRB at 36°C and [Ca$^{2+}$]$_i$ was measured by dual-wavelength fura-2 microfluorometry with excitation at 340/380 nm and emission at 510 nm by using a cooled charge-coupled device camera. Fluorescence ratio images were produced using an Aquacosmos system (Hamamatsu Photonics, Hamamatsu, Japan). Cells used for single-cell experiments fulfilled the morphological and physiological criteria for insulin-positive β-cells, including the diameter and
responsiveness to glucose and tolbutamide. Effects of GLP-1 on $[Ca^{2+}]$, were investigated exclusively in the cells that responded to glucose with increases in $[Ca^{2+}]$, in a β-cell–specific manner and to tolbutamide at the end of recording.

**Electrophysiological experiments**

Perforated whole-cell currents were recorded using a pipette solution containing amphotericin B (200 µg/ml) dissolved in 0.1% DMSO. Membrane currents, recorded by using an amplifier (200B; Axopatch, Foster, CA, USA), were stored online in a computer with pCLAMP10.2 software. Voltage clamp in perforated mode was considered to be adequate when the series resistance was <20 MΩ. Patch pipettes purchased from Narishige (Tokyo, Japan) and their resistances ranged from 3 to 5 MΩ when filled with pipette solution that contained 40 mM K$_2$SO$_4$, 50 mM KCl, 5 mM MgCl$_2$, 0.5 mM EGTA, and 10 mM HEPES at pH 7.2 with KOH. To record the background current, β-cells were voltage-clamped at a holding potential of -70 mV or -80 mV. In the presence of 100 µM tolbutamide, which is a sufficient concentration to specifically block K-ATP channels, the residual current is background current corresponding to NSCC conductance. Membrane potential was stepped to test potentials from -100 to -20 mV every 10 s in the presence of 5.6 mM glucose. After recording
the control current, the bathing solution (HKRB) was changed to a solution containing test agent.

Measurement of membrane potentials was performed by switching from perforated whole-cell voltage-clamp mode to current-clamp mode. We demonstrated that the voltage-clamped cell was immunostained with anti-insulin antiserum and shown to express insulin-positive fluorescence (11). Electrophysiological experiments were performed at 27°C.

**Statistical analysis**

Data are presented as means (standard error of the mean, SEM) and were compared by Student’s t-test performed with GraphPad Prism ver. 5.0. P-values of <0.05 were considered to be statistically significant.
Results

Exendin-4 depolarizes the plasma membrane in association with increasing background current.

In order to examine whether exposure of rat β-cells to GLP-1 depolarizes the membrane and whether the change in membrane potential is a consequence of NSCC activation, membrane potential and whole-cell currents were recorded. During exposure to the GLP-1 receptor agonist, exendin-4 (ex-4) at a concentration of 10^{-10} M at 2.8 mM glucose, the plasma membrane was depolarized (Fig 1A and B). There has been a report suggesting that K-ATP channels are inhibited by exendin-4 (5). To examine effects of ex-4 on NSCC current without influencing changes in activity of the K-ATP channel by ex-4, we voltage-clamped the cell at -70 mV that is close to potassium equilibrium potential and used tolbutamide to inhibit the K-ATP channel. Under these conditions, ex-4 increased the inward background current in β cells (Fig. 1C, Supplementary Fig. 1). When the subtracted currents sensitive to ex-4 were plotted against the membrane voltage for the current-voltage (I-V) relationship, the reversal potential was -19.2 mV, and it was shifted to -4.4 mV (Fig. 1D) by omitting external Ca^{2+}. Thus, the Ex-4-sensitive channel is permeable to Na^{+}, K^{+} and Ca^{2+}. The reversal potentials were consistent with NSCC reversal potentials in previous reports, in which GLP-1 elicited
the NSCC current with reversal potentials ranging from -20 mV to 0 mV (2; 13). The slope conductances in normal and Ca\(^{2+}\)-free HKRB solutions were 82.3 pS/pF and 25.2 pS/pF, respectively. These results indicate that the ex-4-sensitive current is in part Ca\(^{2+}\) permeable (Fig. 1D). Ex-4 increased the current in a concentration-dependent manner; the effect started at 0.01 nM, peaked at 0.1 – 1 nM, and declined at 10 nM and higher (Fig. 2).

Potentiation of first-phase insulin secretion and cytosolic Ca\(^{2+}\) caused by increasing NSCC current via cAMP/EPAC/NSCC pathway by ex-4.

Next, we confirmed that incretin hormone could increase first-phase insulin secretion and [Ca\(^{2+}\)]\(_i\) response to glucose. Insulin secretion measured during 15-min (Fig. 3A-1) or 5-min (Supplementary Fig. 2) static incubation with 16.6 mM glucose was increased by addition of ex-4. Increases in [Ca\(^{2+}\)]\(_i\) is a primary context prior to initiation of insulin secretion. An increase in glucose concentration from 2.8 mM to 5.6 mM induced first-phase increases in [Ca\(^{2+}\)]\(_i\), and a subsequent increase in glucose to 8.3 mM induced further increases in [Ca\(^{2+}\)]\(_i\) in an oscillatory pattern. The [Ca\(^{2+}\)]\(_i\) oscillation declined with time during 20 min of exposure to 8.3 mM glucose under control conditions, while it
was maintained or even enhanced in the presence of GLP-1 (Fig. 3A-2). GLP-1 significantly increased the area under the curve of \([\text{Ca}^{2+}]_i\) oscillations (AUC, Fig. 3A-3).

In order to clarify the mechanistic pathways in which incretin hormone facilitates the opening of NSCC, we tested compounds that mediated effects downstream of receptor stimulation. Both ex-4 and GLP-1, as well as another GLP-1 analog, liraglutide, significantly increased inward current at concentrations of \(10^{-10}\) M measured at \(-70\) mV in \(\beta\)-cells (Fig. 3B, C, D). The \(\text{K}^+\) channel currents could be changed minimally, if at all, at this potential. \(\text{K}^-\text{ATP}\) channels and voltage-gated potassium (Kv) channels are inhibited by 100 \(\mu\)M tolbutamide and are voltage-dependently inactivated, respectively. Therefore, throughout the subsequent experiments, we considered the inward current evoked at \(-70\) mV to be that of NSCC. To further confirm this hypothesis the membrane potential was held at \(-80\) mV, which is closer to potassium equilibrium potential (\(-80\) mV based on calculations from external and internal potassium concentrations, 5.9 mM and 130 mM, respectively, at 27ºC). Ex-4 at \(10^{-10}\) M elicited increases in inward current in the absence of tolbutamide (Supplementary Fig. 3A, B). Tolbutamide was without effect on current increased by ex-4 at \(10^{-10}\) M in the presence of 5.6 mM glucose, and was not able to induce NSCC current at \(-80\) mV (Supplementary Fig. 3C, D). The ex-4-induced NSCC-current increase was attenuated
in the presence of GLP-1 receptor antagonist, ex-(9, 39). This suggests that GLP-1 induces the current increase by interacting with GLP-1 receptor (Fig. 3E, see Supplementary Fig. 4A for current trace).

There are several pieces of evidence indicating that GLP-1 stimulates glucose-induced insulin secretion via cAMP production (14-16). Consistently, exposure to 1 mM dbcAMP evoked an NSCC current increase (Fig. 3F), and the PKA inhibitor H89 (Fig. 3G) did not prevent the increase in NSCC current induced by ex-4. Another potent PKA inhibitor, KT5720 (Supplementary Fig. 4B, C), had no effect on the ex-4-induced current increase in NSCC. The PKA activators 100 µM 6-Benz cAMP (Fig. 3H) and 10 µM 6-Phen cAMP (Fig. 3I) did not influence the amplitude of NSCC current (see Supplementary Fig 4D, E for original current traces). The ex-4-induced NSCC current increase was mimicked by activator of exchange protein directly activated by cAMP (EPAC) (8-pCPT, Fig. 3J). An EPAC inhibitor, (ESI-09; 3-(5-tert-butyl-isoxazol-3-yl)-2- [(3-chlorophenyl)-hydrazono]-3-oxo-propionitrile) (17), attenuated the ex-4-induced NSCC-current increase (Fig. 3K, Supplementary Fig. 5A). Similarly, ex-4 did not increase NSCC current in the presence of RAP1-inhibitor, GGTI-298 (Supplementary Fig. 5B) (18). These data suggest that GLP-1/ex-4-sensitive current is regulated by a cAMP-EPAC2 (or EPAC1) pathway and that RAP1 is involved.
The ex-4-sensitive current increase was prevented by a nonspecific blocker of TRP channels, ruthenium red (not shown), and TRPM2-channel antagonist (2-APB, 2-aminoethyl diphenylborinate; Fig. 3L, Supplementary Fig. 5C) (19). GIP also increased NSCC current (Fig. 3M), and this current increase was attenuated by pretreatment of β-cells with 2-APB (Fig. 3N). GIP dose-dependently increased NSCC current at concentration ranges similar to those of ex-4 with a peak effectiveness observed at 0.1 nM GIP (Fig. 2B). The concentrations of GLP-1 or GIP that affected NSCC were in the suprapicomolar range, which is similar to the physiological plasma levels of these hormones after meal intake (20). Insulin secretion stimulated by 16.6 mM glucose was potentiated by the presence of 0.1 or 1 nM ex-4. This effect of ex-4 on the potentiation of glucose-stimulated insulin secretion was inhibited by 2-APB (Fig. 4).

**NSCC activation is evoked by glucose metabolism and is potentiated by ex-4**

EPAC2 serves as a major pathway for cAMP-mediated potentiation of glucose-stimulated insulin secretion (21). Glucose metabolism elevates cytoplasmic cAMP levels in β-cells (22-24). Hence, we tested whether glucose metabolism influences EPAC-regulated NSCC. We found that an increase in glucose concentration to 16.6 mM reversibly increases the NSCC current, and the I-V relationship showed that
the reversal potential and slope conductance were -15.4 mV and 82.6 pS/pF, respectively (Fig. 5A, B). These properties were consistent with those of the NSCC current induced by ex-4. Glucose at 2.8, 5.6 and 16.6 mM dose-dependently increased the NSCC current, and addition of ex-4 at each glucose concentration increased the current (Fig. 5C). These effects of glucose on current increases in NSCC were not the result of exposure to tolbutamide, as NSCC current was evoked by increasing glucose concentration to 16.6 mM in the absence of tolbutamide and addition of tolbutamide did not influence the current level (Supplementary Fig. 3E, F). The amplitude of the current increase by ex-4 was greatest at 5.6 mM and was lesser at 16.6 mM glucose. Exposure of β-cells to an uncoupler of electron transport in mitochondria, 3-trifluoromethoxyphenylhydrazone (FCCP), or 2-APB attenuated the glucose-induced NSCC-current increase. Sucrose (13.8 mM) did not mimic the high glucose-induced NSCC-current increase (Fig. 5D, Supplementary Fig. 6A, B). These results suggest that increased glucose metabolism and GLP-1/GIP receptor stimulation elicit increased activity in the same type of NSCC.

Attenuation of glucose, ex-4 and GIP–elicited increase in NSCC current in β-cells

from TRPM2 KO mice
In order to examine whether TRPM2 channels are involved in ex-4 and glucose-induced NSCC-current increases, effects of these stimuli on NSCC current were studied in TRPM2-deficient mice. Ex-4, high glucose (Supplementary Fig. 7A), GIP and 8-pCPT increased NSCC current to a similar degree as in wild-type mice (Fig. 6A, Supplementary Fig. 7A, 8A). In TRPM2-KO mice (8; 12), these effects were blunted (Fig. 6B, Supplementary Figs. 7B, 8B). The AUC of [Ca\(^{2+}\)]\(_i\) response during exposure to 8.3 mM glucose was attenuated in TRPM2 KO mice (Supplementary Fig. 9). The TRPM2 channel is a major physiological target of glucose metabolism-evoked and incretin hormone–potentiated insulin secretion.

8-pCPT-AM, an activator of EPAC with little activation effect on PKA at lower micromolar doses (25), enhanced the NSCC-current increase at 2.8 mM glucose more potently than ex-4 (Fig. 6C). Increasing glucose to 16.6 mM did not overwhelm 8-cCPT-AM stimulation of NSCC at 2.8 mM. ESI-09 added to 16.6 mM glucose inhibited the increasing effects of glucose on NSCC. Accordingly, the pathway of EPAC activation to TRPM2 channel opening is commonly utilized by glucose metabolism and GLP-1 in these two important stimuli on insulin secretion.

Co-stimulation with 16.6 mM glucose and tolbutamide induced greater depolarization than 2.8 mM glucose and tolbutamide (Fig. 6D). The membrane potentials were
changed from \(-66.0 \pm 1.6\) mV (left white bar) at 2.8 mM glucose to \(-18.2 \pm 1.8\) mV (left black) at 16.6 mM glucose with tolbutamide (100 \(\mu\)M) and from \(-67.0 \pm 0.6\) mV (right white) in control at 2.8 mM to \(-50.4 \pm 0.9\) mV (right black) after addition of tolbutamide.

As tolbutamide-induced depolarization per se did not increase activity of TRPM2 channel (compare amplitudes of white bars in Fig. 6A with corresponding white bars in Fig. 6B), high-dose glucose in the presence of tolbutamide produces greater depolarization than low-dose glucose with tolbutamide, presumably by activating NSCC (TRPM2) as an additional enhancing signal. These results were further confirmed in wild-type and TRPM2 KO mice (Supplementary Fig. 10). We observed that depolarization by tolbutamide was further enhanced after addition of EPAC activator, 8-pCPT-AM, at 2.8 mM glucose in wild-type mice. This additional depolarization by 8-pCPT-AM was attenuated in TRPM2 KO mice.
Discussion

Both glucose metabolism and GLP-1 receptor stimulation increased the activity of TRPM2 channels via the cAMP-EPAC pathway but not the PKA pathway. As increases in glucose concentration reportedly induce oscillations of cAMP in the cytoplasmic space in β-cells and these oscillations are preceded and potentiated by elevation of [Ca$^{2+}$], (24), the activation of the cAMP/EPAC/TRPM2 channel by glucose metabolism may further facilitate glucose-induced depolarization. Although the depolarization initiated by glucose metabolism is believed to simply be a consequence of closure of the K-ATP channel, we now propose that the depolarization is a clear consequence of the simultaneous occurrence of both K-ATP channel closure and TRPM2 channel opening. Ex-4 or the EPAC activator, 8-pCPT-AM inhibited K-ATP channels in whole-cell and inside-out patch experiments (5). Thus, ex-4 may stimulate β-cells cooperatively by inhibiting K-ATP channels and activating TRPM2 channels.

The glucose-induced membrane depolarization results from not only closure of the K-ATP channel but also opening of TRPM2 channels. Exposure to tolbutamide at 2.8 mM glucose depolarized to -50.4 mV (Fig. 6D, right black bar), whereas it was more depolarized to -18.2 mV at 16.6 mM glucose (left black bar). Most K-ATP channels are opened at 2.8 mM glucose and a small amount of TRPM2 channels are opened (Fig.
The low-level openings of TRPM2 channels led membrane depolarization by only 17 mV after addition of tolbutamide (right bars in Fig. 6D). In contrast, when we used high glucose concentration (left bars in Fig. 6D), membrane was further depolarized by tolbutamide. As TRPM2 channels are further opened in high glucose as demonstrated in Fig. 5C, simultaneous stimulations with high glucose and tolbutamide are more effective for depolarization than tolbutamide alone at low glucose. In wild-type mice 8-pCPT-AM further depolarized the membrane after addition of tolbutamide but not in KO mice. Activation of EPAC depolarizes the membrane through increases in activity of TRPM2 channels.

We have observed current increases in TRPM2 channels by ex-4 and high glucose regardless of the presence of tolbutamide. Similarly, amplitudes of currents without ex-4 in wild and KO mice were not different despite tolbutamide was present in both (white bars in Fig. 6A and B). These results suggest that tolbutamide alone did not influence TRPM2 channels at 2.8 mM and 16.6 mM glucose. Recently, EPAC2A was found to be a central mediator in GLP-1-stimulated insulin secretion (26). It is likely that TRPM2 is regulated by EPAC in our study. Interaction of SUR1/EPAC2A protein-protein (27-29) and increased EPAC2A activity by sulfonylureas (30) has been reported. PKA-independent potentiation of insulin secretion by cAMP (presumably
EPAC2A-dependent pathway) was attenuated in SUR1-deficient islets (31). The functional relationship between these proteins, sulfonylureas and TRPM2 channels remains to be elucidated. The observation that TRPM2 channels could not be activated by ex-4 in the presence of 10 µM GGTI-298, RAP1 inhibitor suggests direct or indirect interaction of TRPM2 channels with RAP1 downstream of EPAC. The reasons of the difference between our results showing ineffectiveness of tolbutamide on TRMP2-channel current (Fig. 6A, B; white bars and Supplementary Fig. 3) and the increase in activity of EPAC2A by sulfonylureas including tolbutamide (30; 32) are unknown. It has been proposed that there are two binding sites for cAMP corresponding to high and low affinity sites for cAMP in EPAC2A (30). Sulfonylureas bind to low affinity site. If intrinsic cAMP levels produced by ex-4 or glucose are high enough to bind to both sites, tolbutamide can no longer bind to the low affinity site. Under these conditions, tolbutamide may not influence TRPM2 channels stimulated by ex-4 or glucose. We used tolbutamide at 100 µM that may be insufficient concentration to activate TRPM2. EPAC1 does not have binding site to sulfonylureas (30). Further investigations should be warranted.

The changes in TRPM2-channel current demonstrated in the present study might be dependent on indirect or direct protein-protein interactions among
K-ATP/EPACs/TRPM2 that combines in modulation of TRPM2-channel currents. The changes were observed under the conditions of little or no K-ATP channel current, and therefore are not absolutely dependent on K-ATP channel current. Changes in protein-protein interactions of the K-ATP/EPACs/TRPM2 proteins could contribute to the results observed in the absence of K-ATP channel currents.

Adenosine 5’-diphospho-ribose (ADPR), cyclic ADP-ribose (cADPR), and H$_2$O$_2$ are potent activators of TRPM2 (19; 33). GLP-1 reportedly produces cADPR in mouse pancreatic islets (34), suggesting that cADPR plays a key role between EPAC and TRPM2 activation. Although TRPM2 activation by cADPR is dependent on temperature (>35°C) (33) and we performed the present experiments at 27°C, further investigation is required to elucidate whether cADPR is involved in the TRPM2-channel openings demonstrated in the present study.

In the present study, I-V relationship revealed that the GLP-1 and glucose-evoked current reversed around -20 mV, although the I-V relationship of TRPM2 channels expressed in HEK293 cells showed a reversal at 0 mV in both single channel and whole-cell analyses (33). The difference between these zero current potentials may be due to the distinct cell types, β-cell and HEK293 cell, and recording mode, perforated whole-cell clamp. HEK293 cells have a small amount of background current and
voltage-dependent current system. Thus, in these cells, expressed TRPM2 channels should reverse at 0 mV because of its non-selectivity for cations. Similarly, as the TRPM2 channel also is permeable to Ca\textsuperscript{2+}, an increase of Ca\textsuperscript{2+} influx through activated TRPM2 channels may cause an elevated cytosolic Ca\textsuperscript{2+} concentration in a local submembrane area. As a consequence of changes in cytosolic Ca\textsuperscript{2+} concentration due to weak buffer capacity in the perforated whole-cell mode, the Ca\textsuperscript{2+}-activated and voltage-gated potassium channel (BK channel) may influence whole-cell current during glucose or GLP-1 stimulation. These effects may shift the reversal potential toward a negative direction upon TRPM2-channel activation by glucose and/or GLP-1. This hypothesis can be supported by the finding that I-V relations reversed at -4.4 mV in the absence of external Ca\textsuperscript{2+} (Fig. 1D).

The effects of ex-4 and GIP on TRPM2-channel current with regard to dose-responsiveness showed a bell-shaped relationship (Fig. 2). The reduced effectiveness at these high doses of ligand can be explained by desensitization of the G-protein-coupled receptor (GPCR) signal (35). However, the effectiveness of the ligands on TRPM2-channel current was not zero, even in the nanomolar range. Thus, desensitization is not complete.
Our results show that glucose metabolism not only closes K-ATP channels but opens TRPM2 channels and that these pivotal phenomena occur cooperatively. The two channel modulations work in concert to effectively depolarize the plasma membrane, serving as the triggering mechanism (Fig. 7). Stimulation of β-cells by GLP-1 or GIP potentiates glucose-induced TRPM2-channel openings resulting in easier and greater depolarization of the plasma membrane. β-cells may be primed by the incretin-stimulated TRPM2 activation for glucose-induced insulin secretion, as the time required from the beginning of glucose or ex-4 stimulation to TRPM2-channel opening is rapid (<2 min) (Fig. 1A and 5A). The present findings place TRPM2 in the glucose and GPCR signaling pathway in β-cells. TRPM2 channels as a novel partner with K-ATP channels are essential for priming, triggering β-cell insulin secretion. The present study, therefore, suggests TRPM2 as a potential target for the treatment of type 2 diabetes.
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Figure legends

**Fig. 1.** Depolarization of the membrane potential in association with increased background current induced by exendin-4 (ex-4). (A) The membrane potential of a β-cell isolated from rat islets was recorded at 2.8 mM glucose. The membrane was depolarized upon superfusion with $10^{-10}$ M ex-4 in a reversible manner. (B) Comparison of resting membrane potentials in the absence and presence of $10^{-10}$ M ex-4. Glucose concentration was 2.8 mM. Membrane potentials in the absence and presence of ex-4 were $-64.2 \pm 1.3$ mV and $-58.4 \pm 2.1$ mV, respectively. * P<0.01 n=9. (C) Perforated whole-cell clamp experiment performed in the absence (control) and presence of $10^{-10}$ M ex-4 showed that ex-4 increased inward currents (see subtracted currents that were current components increased by ex-4). (D) Mean current levels during test pulses of subtracted current traces were measured and plotted against corresponding voltage. The I-V relationship showed a slope conductance of 82.3 pS/pF (95% confidence intervals, CI; 67.3, 97.3) and a reversal potential of $-19.2$ mV (CI; -25.2, -11.0, n=6, closed circles) in the control; in the absence of Ca$^{2+}$, the slope conductance was 25.2 pS/pF (CI; 12.0, 38.4) and the reversal potential was $-4.4$ mV (CI; -22.5, 48.5, n=6, open circles). Lines were drawn by using linear regression fit to the data points. Data are expressed as
mean ± SEM. Tolbutamide at 100 µM was superfused in the experiments shown in (C) and (D) to inhibit K-ATP channels.
Fig. 2. Dose-dependent increases of ex-4- (A) and GIP- (B) sensitive NSCC currents. The current was recorded according to the same protocol demonstrated in Fig. 1C and D. The membrane potential was held at -70 mV and the glucose concentration was 5.6 mM throughout the experiments. The current was expressed by the current density (pA/pF). Data were collected from 5-9 data points in each experiment.

Fig. 3. Ex-4 (10^{-10} M) increases the first-phase insulin secretion evoked by 16.6 mM glucose with increased Ca^{2+} influx and increased NSCC current via the cAMP/EPAC/NSCC pathway. (A-1) ex-4 increased the insulin secretion at 16.6 mM glucose. *: P<0.05 vs. 16.6 mM glucose without ex-4. (A-2) GLP-1 at 10^{-10} M increased the duration of Ca^{2+} oscillations. At the end of the experiment, 100 µM tolbutamide was added to confirm that the responsive cells were β-cells. (A-3) AUC of oscillatory Ca^{2+} responses at 8.3 mM glucose was increased by GLP-1. The results (A-2 and 3) were from mouse β-cells. HKRB solution with 0.1% BSA was used for insulin secretion or Ca^{2+} measurements. (B-N) Effects of the following agents on current are shown: GLP-1
(B), liraglutide (C) and ex-4 (D) at 10 pM, 10 µM ex-(9-39) (E), dbcAMP (1 mM) (F), H89 (10 µM) (G), PKA activators 6-Benz cAMP (100 µM) (H) and 6-Phen cAMP (10 µM) (I), EPAC activator, 8-pCPT-2-O-Me-cAMP (8-pCPT, 10 µM) (J), EPAC inhibitor (ESI-09; 10 µM, K), TRPM2 inhibitor, (2-APB; 10 µM, L and N) and 10^{-10} M GIP (M). In B, C, D, F, H, I, J, M and N, effects of the indicated agents on NSCC are shown. In E, G, K and L, effects of 10^{-10} M ex-4 on NSCC in the presence of the indicated agents are illustrated. Rat islet β-cells were used and 100 µM tolbutamide was present throughout the experiments of (B) to (N). The number of data points was 6 to 9, and * indicates P<0.05 vs. control (cont) by paired test.

**Fig. 4.** Glucose-induced insulin secretion at 11.2 mM and 16.6 mM and potentiation by ex-4 followed by prevention in the simultaneous presence of 2-APB. Batches containing 10 islets in each were incubated for one hour to evoke insulin secretion at indicated glucose concentrations. Ex-4 potentiated insulin secretion at 0.1 nM and 1 nM. Further addition of 2-APB at 10 µM inhibited ex-4-induced potentiation of insulin secretion. **: P<0.01 vs. 16.6 mM glucose and #: P<0.05 vs. 2-APB by unpaired test.
**Fig. 5.** NSCC activation is evoked by glucose metabolism and potentiated by ex-4.

(A) The holding current at -70 mV was increased by changing the glucose concentration from 2.8 mM to 16.6 mM in the continuous presence of 100 μM tolbutamide. The current recording was interrupted as revealed by IV-a and IV-b for recording I-V relationship. The inwardly increased current with high-dose glucose was reversed when the glucose concentration was changed back to 2.8 mM. Current traces indicated in IV-a and IV-b were evoked by the same voltage protocol described in Fig. 1. Subtracted current traces are depicted (IV-b minus IV-a; IV-c). Current zero level is indicated by 0 with a short bar above the continuous current trace. (B) The I-V relations of subtracted currents was plotted by measuring mean subtracted currents as mentioned in Fig. 1D. The slope conductance was 82.6 pS/pF (CI; 65.3, 99.9, n=6), and the zero current potential was -15.4 mV (CI; -22.6, -4.86, n=6). (C) The glucose-induced NSCC increase was glucose-concentration dependent, and addition of 10^{-10} M ex-4 increased the current. (D) Glucose-induced inward current was attenuated by the presence of FCCP (1 μM) and 2-APB (10 μM). Replacement of glucose with 13.8 mM sucrose did not mimic the glucose-induced inward current increase. Experiments were performed in rat β-cells. *
indicates P<0.05. The number of experiments was 6-9. In C and D 100 µM tolbutamide was present.
Fig. 6. Attenuation of glucose, ex-4 and GIP-induced increases in NSCC current in β-cells from TRPM2 KO mice. (A) Ex-4 (10^{-10} M), 16.6 mM glucose, GIP (10^{-10} M) and 8-pCPT (10 µM) elicited NSCC-current increase in wild-type β-cells. (B) NSCC-current increases by the compounds described above were not observed in TRPM2-KO mice. (C) EPAC pathway serves as glucose- and ex-4–induced NSCC-current increases that were evoked by ex-4 at 10^{-10} M, 10 µM 8-pCPT-AM at 2.8 mM glucose. In the presence of 8-pCPT-AM, increases in the glucose concentration to 16.6 mM did not exaggerate the current amplitude, and 10 µM ESI-09 attenuated the current increase with high glucose. Membrane potential was held at -70 mV and tolbutamide at 100 µM was continuously present when the current was compared between control and tested compounds in (A) to (C). (D) High glucose concentration (16.6 mM) as compared to 2.8 mM glucose induces more depolarization despite the presence of the same concentration of tolbutamide (tolb, 100 µM). Nitrendipine at 10 µM was added to suppress the voltage-dependent Ca^{2+} channels for stable recording of resting membrane potential without induction of electrical firing. *, P<0.05 by unpaired
test. Number of experiments was 6-9. In (C) and (D), experiments were performed in rat β-cells.
**Fig. 7.** Schematic model of GLP-1/GIP-induced opening of TRPM2 channels and cooperative facilitation of depolarization with glucose-induced K-ATP channel closure.

Plus indicates positive regulation, and minus indicates negative regulation.
Fig. 1

Diabetes

A

-70 mV

2 min

20 mV

control

ex-4

B

Membrane potential (mV)

control

ex-4

*  

C

control

ex-4

subtracted

0

50 pA

20 msec

D

Voltage (mV)

Current (pA/pF)

-100 -80 -60 -40 -20

0 2 4 6 8

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Fig. 4

Insulin secretion (ng/ml/10 islets/hour) as a function of Glucose (mM), ex-4 (nM), and 2-APB (µM). The graph shows the effects of varying concentrations of these substances on insulin secretion. Statistically significant differences are indicated by ** (p < 0.01) and # (p < 0.05) compared to the control group.

Glucose (mM) | 2.8 | 11.2 | 11.2 | 2.8 | 16.6 | 16.6 | 2.8 | 16.6 | 16.6 | 16.6
---|---|---|---|---|---|---|---|---|---|---
ex-4 (nM) | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0.1 | 0.1
2-APB (µM) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 10

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Fig. 5

**A**

- **2.8** 100 µM Tolb
- 16.6
- 2.8 mM glucose
- 20 pA
- IV-a
- 50 pA
- IV-b
- IV-c
- 100 sec
- 20 m sec
- 100 pA

**B**

- Voltage (mV)
  - -90 -80 -70 -60 -50 -40 -30 -20 -10 10
- Current (pA/pF)
  - -9 -8 -6 -4 -2

**C**

- Glucose (mM)
  - 2.8 +ex-4 5.6 +ex-4 16.6 +ex-4
- Current (pA/pF)
  - 0 -2 -4 -6 -8 -10
  - *

**D**

- FCCP
- 2-APB
- Glucose (mM)
  - 2.8 16.6 2.8 13.8 Sc 2.8 16.6
- Current (pA/pF)
  - 0 -2 -4 -6 -8 -10
  - *

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**Fig. 7**

The figure illustrates the cellular mechanisms involved in insulin secretion, particularly focusing on the role of glucose metabolism and the interplay between various signaling pathways.

- **Glucose** enters the cell through **GLUT2** and is metabolized in the **mitochondria** to produce **ATP** and **pyruvate**.

- **GLP-1, GIP** stimulate **ATP-sensitive K+ channels (K-ATP)** to close, leading to further **depolarization** of the plasma membrane.

- **Ca**$^{2+}$ influx through **TRPM2 channels** is increased, which further **depolarizes** the membrane.

- **Voltage-dependent Ca**$^{2+}$ channels open, allowing **Ca**$^{2+}$ to enter the cell, contributing to **insulin secretion**.

- **ATP** production from glucose metabolism is further regulated by **AC** (adenylyl cyclase) and **PKA** (protein kinase A) via the **cAMP** pathway, influenced by **GLP-1, GIP**.

- **Na**$^{+}$/Ca**$^{2+}$ exchanger** facilitates the exchange of ions, maintaining cellular homeostasis.

This integrated system highlights the complex interplay between glucose metabolism, intracellular signaling, and ion channel dynamics in the regulation of insulin secretion.
Supplementary figures

Supplementary Fig. 1
Exendin-4 at $10^{-10}$ M increased NSCC current in the presence of 5.6 mM glucose and 100 µM tolbutamide. Holding potential was held at -70 mV. Arrow indicates zero current level. This result was from a rat β-cell.
Supplementary Fig. 2

Insulin secretion at glucose concentrations of 2.8 mM, 16.6 mM and 16.6 mM with ex-4. Ten rat islets were incubated for 5 min in each batch. Number of experiments was 9, results are expressed as means ± SEM, and differences were evaluated by ANOVA.
Supplementary Fig. 3.
Effects of ex-4 and high glucose on NSCC currents in the absence of tolbutamide (Tb) at a holding potential of -80 mV. A, B: Ex-4 at 10^{-10} M was superfused at 5.6 mM glucose. C: Ineffectiveness of tolbutamide on ex-4-induced NSCC current. D: Tolbutamide alone did not affect NSCC current at 5.6 mM glucose. E, F: 16.6 mM glucose elicited an increase in NSCC current in the absence of tolbutamide and subsequent addition of tolbutamide did not influence amplitude of the current (E). Number of experiments was 6 in B, D and F. *; P<0.02 vs. control (open bars). Data were obtained from rat β-cells.
Supplementary Fig.4.
Original traces showing effects of ex-(9-39), KT 5720, 6-Benz cAMP and 6-Phen cAMP. A: ex-4 did not increase the current in the presence of ex-(9-39). B: KT5720, PKA inhibitor, did not influence the current increase by ex-4. C: KT5720 at 1 µM was added to the HKRB solution containing 5.6 mM glucose and 100 µM tolbutamide 5 min before exposure to ex-4. Addition of ex-4 increased the current. D and E: Traces showing effects of 6-Benz cAMP or 6-Phen cAMP on NSCC currents. Number of data points was 6. *, P<0.02 vs. control (open bars). Holding potential was -70 mV. Results from rat β-cells are shown.
Supplementary Fig. 5.
Ineffectiveness of ex-4 in the presence of EPAC inhibitor (A), RAP1 inhibitor (B) and TRPM2 inhibitor (C). A: ESI-09 at 10 µM prevented current increase of ex-4. B: RAP1 inhibitor, GGTI-298 (10 µM), counteracted current increases by ex-4. C: Current increase by ex-4 was also inhibited by 2-APB (10 µM). Holding potential was -70 mV. GGTI-298 was preincubated in cultured β-cells for 12 hours before use and it also was superfused during recording data at the same concentration. Results from rat β-cells are shown.
Supplementary Fig. 6.
Original traces showing effects of 16.6 mM glucose in the presence of FCCP (A) and 2-APB (B). Holding potential was -70 mV. Results from rat β-cells are shown.
Supplementary Fig. 7.
Current traces recorded in wild-type (A) and TRPM2 KO (B) mice and effects of 16.6 mM glucose.
In KO mice, high glucose did not increase TRPM2-channel current. Holding potential was -70 mV.
Supplementary Fig. 8.
Current traces in wild-type (A) and KO (B) mice testing effects of 8-pCPT. Holding potential was -70 mV.
Supplementary Fig. 9.
AUC (area under the curve of [Ca^{2+}]_{i} response to 10^{-10} M GLP-1) in islets isolated from wild-type (Wt) and KO mice.
Means ± SEM are shown and were compared by ANOVA. Glucose concentration was 8.3 mM. AUC was measured from [Ca^{2+}]_{i} response during 20-min exposure of cells to 8.3 mM glucose.
Supplementary Fig. 10
Depolarization by EPAC activator is mediated by TRPM2 channel.
Membrane potentials were measured in control (white bars; -68.0 ± 1.4 mV in WT, -70.0 ± 1.0 mV in KO), in the presence of 100 µM tolbutamide (black bars; -29.0 ± 1.8 mV in WT, -26.3 ± 1.7 mV in KO) and in the presence of tolbutamide + 10 µM 8-pCPT-AM (red bars; -19.9 ± 1.1 mV in WT, -25.6 ± 1.5 mV in KO) in β-cells from wild-type and KO mice. *, P<0.01 vs. tolbutamide.