A novel mechanism of imeglimin-mediated insulin secretion via the cADPR-TRP channel pathway

Shunsuke Funazaki1, Masashi Yoshida1*, Hodaka Yamada1, Masafumi Kakei1, Masanobu Kawakami1, Shuichi Nagashima1, Kazuo Hara1, Katsuya Dezaki2,3

1Department of Medicine, Division of Endocrinology and Metabolism, Jichi Medical University Saitama Medical Center, Saitama, Japan, 2Department of Physiology, Division of Integrative Physiology, Jichi Medical University, Shimotsuke-shi, Japan, and 3Faculty of Pharmacy, Iryo Sosei University, Iwaki, Japan

Keywords
Cyclic ADP ribose, Imeglimin, Transient receptor potential melastatin 2

*Correspondence
Masashi Yoshida
Tel.: +81-48-647-2111
Fax: +81-48-648-5166
E-mail address: myoshida-md@jichi.ac.jp

Katsuya Dezaki
Tel.: +81-246-29-5372
Fax: +81-246-29-5372
E-mail address: dezaki@jichi.ac.jp; dezaki.katsuya@isu.ac.jp

ABSTRACT

Aims/Introduction: Imeglimin is a novel oral hypoglycemic agent that improves blood glucose levels through multiple mechanisms of action including the enhancement of glucose-stimulated insulin secretion (GSIS), however, the details of this mechanism have not been clarified. In the process of GSIS, activation of the transient receptor potential melastatin 2 (TRPM2) channel, a type of non-selective cation channel (NSCCs) in β-cells, promotes plasma membrane depolarization. The present study aimed to examine whether imeglimin potentiates GSIS via the TRPM2 channel in β-cells.

Materials and Methods: Pancreatic islets were isolated by collagenase digestion from male wild-type and TRPM2-knockout (KO) mice. Insulin release and nicotinamide adenine dinucleotide (NAD+) production in islets were measured under static incubation. NSCC currents in mouse single β-cells were measured by patch-clamp experiments.

Results: Batch-incubation studies showed that imeglimin enhanced GSIS at stimulatory 16.6 mM glucose, whereas it did not affect basal insulin levels at 2.8 mM glucose. Imeglimin increased the glucose-induced production of NAD+, a precursor of cADPR, in islets and the insulinotropic effects of imeglimin were attenuated by a cADPR inhibitor 8-Br-cADPR. Furthermore, imeglimin increased NSCC current in β-cells, and abolished this current in TRPM2-KO mice. Imeglimin did not potentiate GSIS in the TRPM2-KO islets, suggesting that imeglimin’s increase of NSCC currents through the TRPM2 channel is causally implicated in its insulin releasing effects.

Conclusions: Imeglimin may activate TRPM2 channels in β-cells via the production of NAD+/cADPR, leading to the potentiation of GSIS. Developing approaches to stimulate cADPR-TRPM2 signaling provides a potential therapeutic tool to treat type 2 diabetes.

INTRODUCTION

Imeglimin, with the chemical name (6R)-(+)-4,4-dimethylamino-2-imino-6-methyl-1,2,5,6-tetrahydro-1,3,5-triazine hydrochloride, is the first member of the oral tetrahydrotriazine-containing compounds, a glimin. It was originally developed to be used in combination therapies in addition to other drugs to improve insulin secretion and susceptibility in patients with type 2 diabetes mellitus1-3. Detailed studies have shown that this drug may have a positive effect on metabolism by improving glucose and lipid profiles in the context of diabetes4-6. Imeglimin is currently under clinical trials around the world and has been reported to improve both blood glucose and glycated hemoglobin (HbA1c) levels and to be safe4,6.

Evidence emerging from in vitro and in vivo studies suggests that imeglimin has a potent anti-hyperglycemic effect and can normalize glucose homeostasis via several pathways including improving mitochondrial function3, maintaining β-cell function, and increasing glucose-induced insulin secretion5,7. Hallakou-Bozec and colleagues have recently proposed that imeglimin...
stimulates insulin secretion by activating the salvage pathway in a dose-dependent manner increasing the nicotinamide adenine dinucleotide (NAD\(^+\)) pool; major metabolites of cyclic ADP-ribose (cADPR), are also involved in the dose-dependent mechanism.\(^8\)

The transient receptor potential melastatin 2 (TRPM2) channel is one of the nonselective cation channels (NSCC) and is expressed in pancreatic \(\beta\)-cells.\(^9\) It has been long suggested that glucose-induced insulin secretion in pancreatic \(\beta\)-cells was assumed to be caused by the closure of ATP-sensitive \(K^+\) (KATP) channels and thereby depolarization of the membrane.\(^10\) Theoretically, the closure of KATP channels is an essential process for glucose-induced insulin secretion, but it is not sufficient to induce a shift in membrane potential to a threshold level, because the membrane potential is determined by the overall balance of outward and inward currents.\(^11\) During the initiation of insulin secretory stimulation in pancreatic \(\beta\)-cells, glucose-stimulated opening of NSCC results in increased background currents and increased \(Na^+\) and \(Ca^{2+}\) influx into the cell.\(^11\) With respect to the regulation of the resting membrane potential, dual channel-regulation by KATP channels and NSCC is important, as this phenomenon works in conjunction with the increased membrane resistance due to the closure of KATP channels to promote plasma membrane depolarization.\(^12\) We have demonstrated that the presence of TRPM2 plays an important role in \(\beta\)-cell depolarization by GLP-1 and glucose,\(^13\) while adrenaline suppresses background currents, prolongs latency to depolarization, and reduces \(\beta\)-cell glucose responsiveness.\(^14\) It has been suggested that cADPR may be involved in the GSIS of imeglimin due to its importance in the mechanism of insulin secretion via TRPM2 channels through its function as a TRPM2 promoter.\(^15\) However, the relationship between imeglimin and the cADPR-TRPM2 pathway and the involvement of imeglimin in phase 1 insulin secretion are unknown. In order to clarify these mechanisms, we conducted several experiments using male C57BL/6J mice and TRPM2-knockout (KO) mice with reduced phase 1 insulin secretion for comparison.\(^16\)

**MATERIALS AND METHODS**

**Preparation of islets and single pancreatic \(\beta\)-cell**

Male C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) and TRPM2-KO mice were used according to the institutional guidelines and the Physiological Society of Japan’s animal husbandry guidelines for a 12 h light/dark cycle. TRPM2-KO mice were kindly provided by Dr Y. Mori (Kyoto University).\(^17\) TRPM2-KO mice were backcrossed with the C57BL/6J strain for at least nine generations. They were kept in an air-conditioned room, and food and water were freely available. Islets of Langerhans were isolated by collagenase digestion from male C57BL/6J and TRPM2-KO mice (aged 8–12 weeks) using a previously reported method.\(^18,19\) After anesthesia by intraperitoneal injection of pentobarbital (100 mg/kg), collagenase 1.05 mg/mL in Krebs-Ringer bicarbonate buffer (HKRB) supplemented with HEPES containing 5 mM CaCl\(_2\) was injected directly into the common bile ducts. The composition of HKRB was 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\(_4\), 10 mM HEPES, pH 7.4, NaOH. The pancreas was dissected and incubated in HKRB buffer for 15 min at 37°C. Size-matched islets were harvested and used for insulin and dinucleotide release experiments in static incubation. In addition, electrophysiological experiments were performed using single \(\beta\)-cells dispersed in Ca-free HKRB 0.01% bovine serum albumin (fatty acid free; purchased from Sigma-Aldrich). The HKRB was used to measure insulin secretion. All experimental protocols for animal experiments were conducted according to and approved by the Institutional Committee on Animal Care of Jichi Medical University. Every effort was made to minimize the suffering.

**Measurement of insulin secretion and NAD\(^+\) production from mice islets**

Each batch of 10 size-matched islets was placed in HKRB with 2.8 mM glucose for stabilization for 30 min at 37°C, and then incubated in HKRB with 2.8 mM or 16.6 mM glucose with and without imeglimin (100 \(\mu\)M; Adooq Bioscience LLC, USA) for 10 min to evaluate the first phase of insulin secretion, and 60 min to evaluate the late phase of insulin secretion. Microtubes were centrifuged in a temperature of 4°C at 700 rpm for 15 s, and the supernatant of each microtubde was collected and measured for insulin concentration using ELISA kits (Morinaga Institute of Biological Science, Yokohama, Japan). Other compounds were added to test culture batches at various concentrations, as described below. 2-APB (10 \(\mu\)M; Cayman Chemical, Ann Arbor, MI, USA) was used as a TRP channel blocker; 8-bromo-cyclic adenosine diphosphate ribose (8-Br cADPR; 100 \(\mu\)M; Santa Cruz Biotechnology, Inc. Dallas, TX, USA) was used as a cADPR inhibitor. After 10 min incubation in HKRB with 2.8 or 16.6 mM glucose with imeglimin and 2-APB, 8-Br cADPR secreted insulin. The dinucleotide content was determined with 10 islets/microtube; islets were placed in HKRB with 16.6 mM glucose with or without imeglimin for 60 min. The supernatants were removed by centrifugation and the islets were stored at –80°C followed by lysis in PBS dodecyltrimethylammonium bromide solution; NAD\(^+\) was quantified by a luciferase assay provided in the NAD\(^+\)/NADH Glo assay kit (#G9071, Promega, Madison, WI, USA).

**Patch-clamp experiments**

Whole cell currents perforated were recorded using a pipette solution containing amphotericin B (200 mg/mL) dissolved in 0.1% DMSO. Membrane currents, which were recorded using an amplifier (Axopatch 200B; Axon, Foster, CA), were stored online on a computer with pCLAMP 10.2 software. When the series resistance was under 20 MΩ, the voltage clamp in perforated mode was considered appropriate. Patch pipettes were purchased from Narishige (Tokyo, Japan) and their resistances ranged from 3 to 5 MΩ when filled with a pipette solution.
containing 40 mmol/L K₂SO₄, 50 mmol/L KCl, 5 mmol/L MgCl₂, 0.5 mmol/L EGTA, and 10 mmol/L HEPES at pH 7.2, KOH. To record the background current, the β-cells were voltage-clamped at a holding potential of −70 mV in the presence of 100 μmol/L tolbutamide, which is a sufficient concentration to specifically block the KATP channels; the residual current is a background current corresponding to NSCC conductance as reported previously. Since glucose increases the TRPM2 current in a concentration-dependent manner, the membrane potentials were clamped at sub-stimulatory concentrations of glucose (5.6 mM) in order to measure the actual effects of the drugs. Electrophysiological experiments were performed at 32–37°C. Since β-cells have a wide variety of sizes and insulin secreting abilities, in this experiment, statistical processing was performed using current density (pA/pF) corrected by capacitance.

Statistical analysis
Data are presented as the mean ± standard error of the mean. Statistical analyses were performed by paired or unpaired Student’s t-test with GraphPad Prism 7.0 software. Values of P < 0.05 were considered statistically significant.

RESULTS
Enhancement of GSIS by imeglimin
The effects of imeglimin on insulin release in mouse-isolated islets were measured under basal (2.8 mM) and stimulatory (16.6 mM) glucose conditions. Insulin release from islets under batch-incubation conditions was greater with 16.6 mM glucose than with 2.8 mM glucose. The early and late phases of glucose (16.6 mM)-induced insulin release were both enhanced significantly by imeglimin (100 μM), whereas imeglimin did not affect basal insulin secretion at 2.8 mM glucose (Figure 1).

Imeglimin potentiates GSIS via activation of TRPM2 channel
We examined whether the activation of the TRPM2 channel was involved in imeglimin-induced enhancement of insulin release. The imeglimin-potentiated insulin release was attenuated by a TRPM2 channel blocker 2-APB (10 μM) (Figure 3a). In islets isolated from TRPM2-KO mice, 16.6 mM glucose induced insulin release was significantly lower than in wild-type mice, whereas basal insulin release at low glucose (2.8 mM) remained unchanged. Imeglimin did not enhance 16.6 mM glucose-induced insulin release in the TRPM2-KO islets (Figure 3b). To test the effects of imeglimin on the NSCC current, we voltage-clamped the cells at −70 mV, which is close to the potassium equilibrium potential, and used tolbutamide to inhibit the KATP channel at 5.6 mM glucose.

Imeglimin caused a significant increase of NSCC currents in β-cells isolated from wild-type mice, but not from TRPM-KO mice (Figure 4a–d). In the absence of imeglimin stimulation, there was no change in the current in either the wild type or knockout mouse (Figure S1). These results suggest that the

Figure 1 | Imeglimin enhanced insulin secretion at supra threshold concentrations of glucose. Ten size-matched mouse islets were placed in each batch and incubated for 10 min under (a) 2.8 mM glucose and (b) 16.6 mM glucose, and (c) for 60 min under 16.6 mM glucose. The number of data points was 4–7. n.s., not significant. †P < 0.005, vs 16.6 mM glucose, by unpaired t-test.
insulinotropic action of imeglimin is mediated by potentiating NSCC currents through TRPM2 channels.

DISCUSSION
Imeglimin has been established to exert anti-diabetic effects by improving mitochondrial function, inhibiting glucose production, and enhancing glucose-dependent insulin secretion. In this study, we confirmed the glucose-dependent insulinotropic action of imeglimin in that the early and late phases of glucose (16.6 mM)-induced insulin release from isolated islets were both enhanced significantly by imeglimin, whereas it did not affect basal insulin secretion at 2.8 mM glucose. While the details on the mechanism by which imeglimin exhibits GSIS were unavailable, we found that imeglimin increased NSCC current in β-cells. It is well known that background NSCC currents play an important role in pancreatic β-cell insulin secretion. Our group has previously reported the importance of background currents as well as the KATP pathway in insulin secretion of pancreatic β-cells. In addition, TRPC3, a member of the TRP family, contributes to GSIS via phospholipase C (PLC) and protein kinase C (PKC).

**Figure 2** | Insulin secretion effects by imeglimin related NAD⁺ and cADPR. Imeglimin increased NAD⁺ content and NAD⁺ biosynthesis in pancreatic islets of wild-type mice. (a) Each batch containing 10 wild-type mouse islets was incubated for 60 min in 2.8 or 16.6 mM of glucose, and imeglimin was added in 16.6 mM glucose. The number of data points was 5–6. Imeglimin-induced insulin secretion was inhibited by cADPR inhibitors. (b) Each batch containing 10 wild-type mouse islets was incubated for 10 min and insulin secretion was enhanced with 2.8 or 16.6 mM glucose. 100 μM Imeglimin or 100 μM 8-Br-cADPR was added. The number of data points was 5–10. n.s.; not significant. *P < 0.05, vs 16.6 mM glucose, †P < 0.005, vs 16.6 mM glucose, #P < 0.005, vs 16.6 mM glucose with 100 μM imeglimin, by unpaired- t test.

**Figure 3** | Imeglimin-induced insulin secretion was inhibited by transient receptor potential (TRP) channel blockers and in TRPM2-knock out mouse. (a) Each batch containing 10 wild-type mouse islets was incubated for 10 min and insulin secretion was enhanced with 2.8 or 16.6 mM glucose. 100 μM imeglimin or 10 μM 2-APB was added in 16.6 mM glucose. (b) Each batch containing 10 TRPM2 knockout mouse islets was incubated for 10 min and insulin secretion was enhanced with 2.8 or 16.6 mM glucose. 100 μM Imeglimin was added in 16.6 mM glucose. The number of data points was 5–11. n.s.; not significant. †P < 0.005 vs 16.6 mM glucose, ##P < 0.001 vs 16.6 mM glucose with 100 μM imeglimin by unpaired- t test.
TRP proteins function not only as Ca\(^{2+}\) influx channels but also as anchor proteins in the plasma membrane\(^{22,23}\). The decrease in TRPM2 protein may also be due to the inability of pancreatic \(\beta\)-cells to maintain EPAC and SUR1 (proteins of KATP channel), which are necessary for insulin secretion, in their normal positions, resulting in decreased insulin secretion. In the present study, imeglimin did not enhance GSIS in the TRPM2-KO islets, suggesting that the NSCC currents through TRPM2 channels are implicated in imeglimin-potentiated insulin secretion. Uchida et al. performed an oral glucose tolerance test on TRPM2-KO mice and found that insulin secretion was significantly decreased 15 min after glucose loading compared with wild-type mice, suggesting that mechanisms related to mitochondrial metabolism would be partly implicated in the late phase of the secretion. A relationship between mitochondrial metabolism and TRPM2 activity is interesting, and should be elucidated in future.

There are many mechanisms by which imeglimin improves blood glucose levels. Imeglimin enhances mitochondrial function by modulating the activity of complexes I and III, promoting the oxidation of mitochondrial fatty acids, and normalizing the phospholipid composition in the mitochondria of diabetic animals\(^4\). In addition, imeglimin decreases glycosis in the liver by inhibiting phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase)\(^5\). Imeglimin has
also been reported to stimulate pancreatic islets in diabetic rats and to activate NAD\(^+\)-dependent mechanisms and to salvage pathways in a dose-dependent manner. The increase in NAD\(^+\) content by imeglinin had been shown to be due to an increase in NAD\(^+\) synthesis coming from nicotinamide via the salvage pathway and not to the \textit{de novo} tryptophan pathway\(^8\). In the present study, we showed that imeglinin promoted NAD\(^+\) synthesis and that a cADPR inhibitor attenuated the insulinotropic response of imeglinin, suggesting that the mechanism is cADPR-mediated.

cADPR was first isolated as a putative metabolite of NAD\(^+\) and it is known to be a second messenger of calcium (Ca\(^{2+}\)) signaling\(^26\). It has been reported that a glucose-induced increase in cADPR concentration was not observed in Ob/Ob mice, which are diabetic models\(^27\).

CD38, a glycoprotein found on the surface of many immune cells is active as a cADPR-producing enzyme involved in glucose-induced insulin release\(^26\). Transgenic mice overexpressing CD38 show enhanced glucose-induced insulin release, whereas, conversely, CD38 knockout mice display a severe impairment in \(\beta\)-cell function\(^29\). It has been shown that CD38 gene mutations are more common in non-insulin-dependent diabetic patients than in healthy subjects, and that administration of sera from patients with CD38 gene mutations attenuates insulin secretion\(^30\). The mechanism, as reported by Okamoto \textit{et al.}, is ATP-mediated CD38 production inhibiting the hydrolysis of cADPR and increasing intracellular calcium, leading to insulin secretion\(^31\). Glucose-stimulated ATP production has been shown to be reduced in Goto-Kakizaki rats, a spontaneous model of diabetes and in patients with type 2 diabetes\(^32,33\).

In addition, cADPR is a potent opener of TRPM2 channels and has been reported to be involved in the insulin secretion of pancreatic \(\beta\)-cells\(^34\). Recent investigations have demonstrated that there is a binding pocket where cADPR can dock to human TRPM2 channels and that cADPR directly activates human TRPM2 channels\(^34\). There is accumulating knowledge on the linkage between cADPR and TRPM2 channels, and our results suggest that imeglinin manifests GSIS through the cADPR-TRPM2 pathway.

There are several limitations to this study. The fact that cADPR promotes Ca\(^{2+}\) release is controversial\(^35\), we recognize that the role of cADPR in islets is still open to debate. In assessing the role of mediators in enhancing insulin secretion in pancreatic \(\beta\)-cells, our experiments were limited by the inability to measure levels of cADPR in islets from this diabetic mouse model. In future studies, it will be important to compare the results with those of mice treated with imeglinin and to measure cADPR itself. It is also worthwhile to examine how the opening of TRPM2 channels is affected when CD38-KO mice are treated with imeglinin. Evaluating the involvement of TRPM2 in NAD\(^+\)/cADPR production using TRPM2-KO mice is also interesting.

In this study, we reported that imeglinin enhances insulin secretion. In clinical practice, caution should be exercised when imeglinin is used in combination with sulfonylureas. One randomized controlled trial revealed that imeglinin significantly improved HbA1c compared with placebo\(^36\). However, no study has compared it with sulfonylureas. In the present study, we demonstrated that imeglinin contributes to enhanced insulin secretion by opening TRPM2. Although the mechanism of action is different, we have reported that GLP-1 opens TRPM2 and enhances insulin secretion\(^13\). Since there are reports that incretin-related drugs increase the risk of hypoglycemia when used in combination with sulfonylureas\(^37\), it is of concern that imeglinin, which is also mediated by the TRPM2 pathway, may also increase the risk of hypoglycemia when used in combination with sulfonylureas. We need to be careful if imeglinin is used simultaneously with sulfonylureas in clinical situations. High-dose sulfonylureas and imeglinin may induce more frequent hypoglycemic episodes.

In conclusion, our results suggest that the TRPM2 channel-mediated mechanism plays an important role in imeglinin-induced glucose-dependent insulin secretion. The results of this study provide new insights into the first and late phase of insulin secretion of imeglinin, and the discovery of this pathway may lead to future signal analysis and new targets for diabetes treatment and the prevention of diabetes development.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Young Scientist (JSPS KAKENHI Grant Number 19K18012), Scientific Research (C) (JSPS KAKENHI Grant Number 18K08525) and Scientific Research (C) (JSPS KAKENHI Grant Number 18K08524) to H.Y., M.Y. and K.D., and JMU Start-Up Grant for Young Investigators to S.F.

DISCLOSURE

The authors declare having no conflicts of interest.

Approval of the research protocol: All experimental protocols for animal experiments were conducted according to and approved by the Institutional Committee on Animal Care of Jichi Medical University (20094-01). Informed consent: N/A.

Approval date of registry and the registration no. of the study: N/A.

Animal studies: All animal experiments were conducted according to and approved by the Institutional Committee on Animal Care of Jichi Medical University (20094-01). Informed consent: N/A.

Animal studies: All animal experiments were conducted following the national guidelines and the relevant national laws on the protection of animals.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** | Nonselective cation channel (NSCC) current in pancreatic β-cells did not differ between wild and TRPM2 knockout mice. (a) Wild and (b) TRPM2 knockout mouse. Each mouse single β-cells were voltage-clamped at –70 mV, under the condition of 5.6 mM glucose and 100 μM tolbutamide throughout the experiments. (c,d) The current density calculated from the average current for 10 s immediately after the start of recording is shown as a white column (0 min), and the current density calculated from the averaged peak current during 3–5 min is shown as a black column (3–5 min). The number of data points was 5. *P < 0.05 by paired-t test.