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The anti-ulcer agent, irsogladine, increases insulin secretion by MIN6 cells

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ABSTRACT

Insulin secretion by pancreatic islets is a multicellular process. In addition to other essential systems, gap junctions are an important component of cell-to-cell communication in pancreatic islets. It is well known that dysfunction of gap junctions causes inappropriate insulin secretion. The anti-ulcer agent, irsogladine, increases gap junctions in some cell types. To examine the effect of irsogladine on insulin secretion, we investigated insulin secretion by MIN6 cells treated with or without irsogladine. The expression of connexin 36 proteins and intracellular cAMP levels were also determined using immunoblotting and ELISA assays, respectively. Irsogladine had no effect on insulin secretion under 5.6 mM glucose conditions. However, under 16.7 mM glucose conditions, irsogladine (1.0×10^{-5} M) induced a 1.7 ± 0.20 fold increase in insulin secretion compared to the control ($P<0.05$). This effect of irsogladine on insulin secretion was inhibited by the addition of the gap junction inhibitor 18-beta-glycyrrhetinic acid. Irsogladine treatment increased the protein level of connexin 36 in the plasma membrane fraction. The intracellular cAMP level in MIN6 cells was significantly, but mildly, increased by irsogladine treatment. Furthermore, Rp-cAMP and H89 inhibited the effects of irsogladine on insulin secretion under high glucose conditions. Irsogladine increases insulin secretion under high glucose conditions. The up-regulation of gap junction channels and the increased level of intracellular cAMP induced by irsogladine treatment suggest that these phenomena are involved in irsogladine-induced increased insulin secretion.

1. Introduction

Adequate insulin secretion by pancreatic islets is required for homeostasis of glucose metabolism. Insulin secretion is a multi-cellular process that involves glucose-dependent metabolic pathways, incretin systems, and an intra-islet communication network (Nlend et al., 2006). Therefore, metabolic control implies tight coordination of the function of the many islets that collectively form the endocrine pancreas. Intra-islet coordination of beta cells involves cell-to-cell communication (Meda et al., 1984; Serre-Beinier et al., 2002), and one of the most important cell-to-cell communication systems is the gap junction (Meda, 1996; Michon et al., 2005). The gap junction is formed by connexon, a hexamer of connexin proteins, which links the cytoplasmic compartments of adjacent cells. Through these channels, cells rapidly exchange cytoplasmic ions and small metabolites that signal the state of activity of neighboring cells (Andreu et al., 1997; Charpantier et al., 2007; Kohen et al., 1979; Kumar and Gilula, 1996; MacDonald and Rorsman, 2006; Mears et al., 1995; Meda et al., 1981, 1983; Moreno et al., 2005; Nlend et al., 2006; Pedersen et al., 2005). In the pancreatic islets, coupling of beta cells is necessary for appropriate insulin secretion (Charollais et al., 2000; Kohen et al., 1983; MacDonald and Rorsman, 2006; Mears et al., 1995; Meda et al., 1990; Nlend et al., 2006; Ravier et al., 2005; Speier et al., 2007).

Connexin 36 (Cx36) is expressed by beta cells, and is the only connexin that can link beta cells (Serre-Beinier et al., 2000; Theis et al., 2004). Alterations in the Cx36 content of insulin-producing cells impair the appropriate insulin secretion in response to specific glucose concentrations (Calabrese et al., 2003; Vozzi et al., 1995). Islets of Cx36 null mice do not display the regular oscillations of intracellular calcium concentration (Ravier et al., 2005; Speier et al., 2007). These islets feature a significantly higher basal release of insulin in the presence of sub-stimulatory glucose concentrations. Islets derived from beta cell-specific Cx36 knockout mice showed not only higher basal, but also impaired glucose-stimulated, insulin secretion (Wellershaus et al., 2008).

The importance of the gap junction for insulin secretion from beta cells prompted us to speculate that certain agents that affect the function of gap junctions may also affect insulin secretion. Our research revealed one such possible agent, irsogladine malate. Irsogladine is used as an anti-gastric ulcer agent and up-regulates gap junctions

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between rabbit gastric epithelial cells (Ueda et al., 1991, 1995) and rat pancreatic acinar cells (Kawasaki et al., 2002). In the present study, we investigated the effects of irsogladine on insulin secretion by mouse insulin-secreting MIN6 cells.

2. Materials and methods

2.1. Chemicals

Irsogladine was provided by Nippon Shinyaku Co. Ltd. (Kyoto, Japan). 18-Beta glycyrrhizinic acid (18-beta-GRA) and H89 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rp-cAMP was purchased from EMD Chemicals Inc. (San Diego, CA, USA). All other routinely used reagents were of analytical or cell-culture grade, and were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries (Osaka, Japan).

2.2. Cell culture

MIN6 cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum and penicillin (100 μ/ml)/streptomycin (100 μg/ml) in a 5% humidified CO_2 atmosphere at 37 °C.

2.3. Measurement of insulin secretion

The insulin secretion assay was performed as described previously (Tokuyama et al., 1997), with some modifications. MIN6 cells were plated into 24-well plates and were tested when they were approximately 80% confluent. The cells were washed in a glucose-free Krebs–Ringer-bicarbonate (KRB) buffer, and were preincubated in KRB buffer with the indicated concentrations of irsogladine or other chemicals for 30 min at 37 °C. The cells were then incubated at each glucose concentration with the indicated concentration of irsogladine or other chemicals for 60 min at 37 °C. At the end of each incubation period, the medium was collected and was immediately stored at −80 °C until insulin was determined using a Mouse Insulin ELISA kit (H type) (AKRIN-011H, Shibayagi, Shibukawa, Japan) according to the manufacturer's instructions.

2.4. Immunoblot analysis

After incubation with or without irsogladine for 30 min, the cells were homogenized in buffer containing 42 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, and a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland), pH 7.6, and were rotated for 1 h at 4 °C. The dissolved fractions were then centrifuged to remove insoluble material. The supernatants were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred onto PVDF membranes. The membranes were blocked in a buffer containing 10 mM Tris–HCl, 150 mM NaCl, 0.2% NP-40 and 4% bovine serum albumin pH 7.5, for 2 h at room temperature. The membranes were then probed with an anti-Cx36 antibody (Invitrogen, Camarillo, CA, USA), anti-beta-actin antibody (Sigma Chemical Co.) or anti-beta-catenin antibody (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 16 h at 4 °C. After the membranes were washed, the blots were incubated with horseradish peroxidase-linked secondary antibody, followed by enhanced chemiluminescence detection using the ECL reagent (Amersham Pharmacia Biotech, Uppsala, Sweden). Films were then exposed and developed and the intensity of the bands was quantified using Image-J software (National Institutes of Health, Bethesda, MD, USA).

The plasma membrane fraction was prepared as described previously (Karnieli et al., 1981; McKeel and Jarett, 1970) with some modifications. Briefly, cells were homogenized using a Dounce tissue grinder in buffer containing sucrose and a protease inhibitor cocktail, followed by sucrose gradient centrifugations to obtain the plasma membrane fractions. The fractions were then dissolved in a buffer containing 42 mM HEPES, 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, and a protease inhibitor cocktail, pH 7.6, and were rotated for 1 h at 4 °C. The dissolved fractions were centrifuged to remove insoluble material. The supernatants were prepared for SDS-PAGE and immunoblot analysis as described above.

2.5. Measurement of intracellular cAMP content

MIN6 cells were cultured in 24-well culture dishes and were treated with or without irsogladine for 20 min. 3-Isobutyl-1-methylxanthine (IBMX; 250 μM) was added to all incubation buffers. Intracellular cAMP concentrations were then determined using the Amersham cAMP Biotrak Enzyme immunoassay (EIA) System (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. Student's *t*-test and one-way ANOVA were used for comparison of data. Values of $P<0.05$ were considered significant. Stat View-J version 5.0 software (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis.

3. Results

3.1. Irsogladine increases insulin secretion by MIN6 cells, an effect requiring gap junctions

To elucidate the effect of irsogladine on insulin secretion, MIN6 cells were treated with several concentrations of irsogladine at two different glucose concentrations. Irsogladine treatment had no effect on insulin secretion by MIN6 cells under 5.6 mM glucose conditions. In contrast, under conditions of 16.8 mM glucose, treatment with 1.0×10^{-5} M irsogladine significantly increased insulin secretion to 1.7 ± 0.20 fold that of the control level ($P<0.05$) (Fig. 1). The effect of irsogladine on insulin secretion occurred in a dose-dependent manner.

To study the contribution of gap junction channels to the increased insulin secretion induced by irsogladine, the effect of a gap junction inhibitor, 18-beta-GRA (Davidson and Baumgarten, 1988; Nlend et al., 2006; Yamamoto et al., 1998), was subsequently assessed. As shown

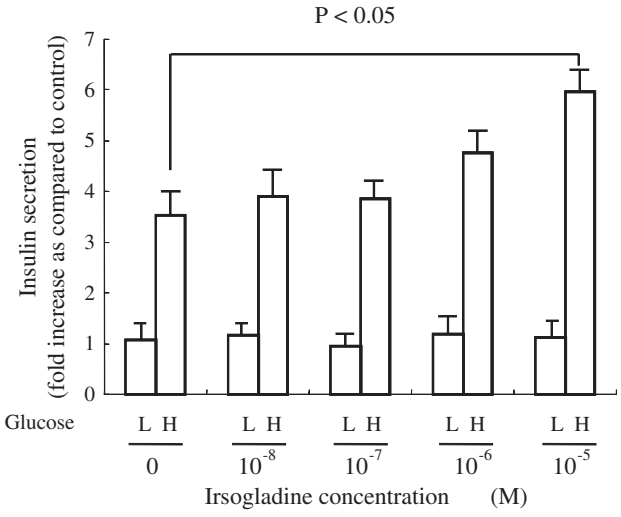

Fig. 1. Effect of irsogladine on insulin secretion by MIN6 cells. MIN6 cells were incubated with or without the indicated concentrations of irsogladine in KRB buffer for 30 min. The cells were then stimulated with 5.6 mM (L) or 16.8 mM (H) glucose for 60 min. Insulin concentrations in the incubation media were measured using an ELISA. Data are shown as means ± S.E.M. (n = 6).

in Fig. 2, the irsogladine-induced increase in insulin secretion was inhibited by the addition of 18-beta-GRA, whereas 18-beta-GRA had no effect on insulin secretion without irsogladine. These data suggest that gap junction channels are involved in the effect of irsogladine on insulin secretion.

3.2. The level of plasma-membrane-associated Cx36 protein is increased by irsogladine-treatment

To determine whether irsogladine modulated the expression level of the Cx36 protein, which is known to be involved in gap junction formation in beta cells, we performed immunoblot analysis of whole cell lysates of MIN6 cells using an anti-Cx36 antibody. Irsogladine treatment did not affect the total protein expression level of Cx36 in MIN6 cells, suggesting that irsogladine could not change the total protein expression of Cx36 in MIN6 cells. However, it has been reported that irsogladine treatment increases the expression level of another connexin specifically in the plasma membrane fraction of cells (Kawasaki et al., 2002). We therefore examined the effect of irsogladine on the plasma membrane-associated expression of Cx36 in MIN6 cells. Irsogladine treatment significantly increased the level of Cx36 protein in the plasma membrane fraction to 2.0 ± 0.12-fold that of the control ($P<0.05$) (Fig. 3).

3.3. Level of intracellular cAMP is up-regulated by irsogladine, cAMP antagonist and PKA inhibitor suppress the effect of irsogladine

Increased intracellular cAMP levels have been reported to stimulate insulin secretion, especially under high glucose conditions (Prentki and Matschinsky, 1987). It has been reported that irsogladine increases intracellular cAMP levels in other types of cells (Ueda et al., 1991). We therefore examined intracellular cAMP levels in MIN6 cells after irsogladine treatment. Irsogladine treatment significantly increased the intracellular cAMP level to 1.3 ± 0.16 fold that of the control ($P<0.05$) (Fig. 4). We next examined the role of intracellular cAMP and protein kinase A, which is activated downstream of cAMP, in the mediation of irsogladine effects, using Rp-cAMP or H89, a protein kinase A inhibitor. Rp-cAMP and H89 inhibited the effects of irsogladine on insulin secretion under high glucose conditions (Fig. 5a, b), suggesting that the cAMP–protein kinase A pathway contributes to the effects of irsogladine on insulin secretion.

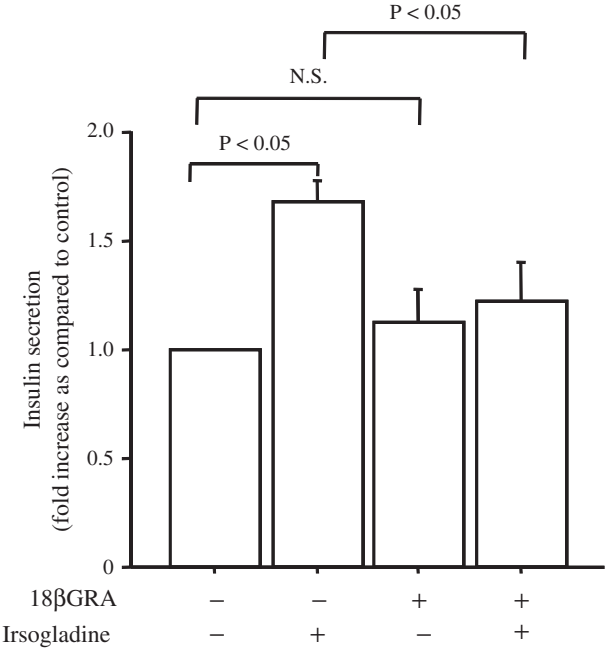

Fig. 2. Effect of a gap junction inhibitor on irsogladine-stimulated insulin secretion by MIN6 cells. MIN6 cells were incubated with or without 1.0×10^{-5} M irsogladine in KRB buffer for 30 min. The cells were then stimulated with 16.8 mM glucose with or without the gap junction inhibitor, 18-beta-glycyrrhizinic acid (18βGRA; 1.0×10^{-5} M), for 60 min. Insulin concentrations in the incubation media were measured using an ELISA. Data are shown as means ± S.E.M. (n = 6).

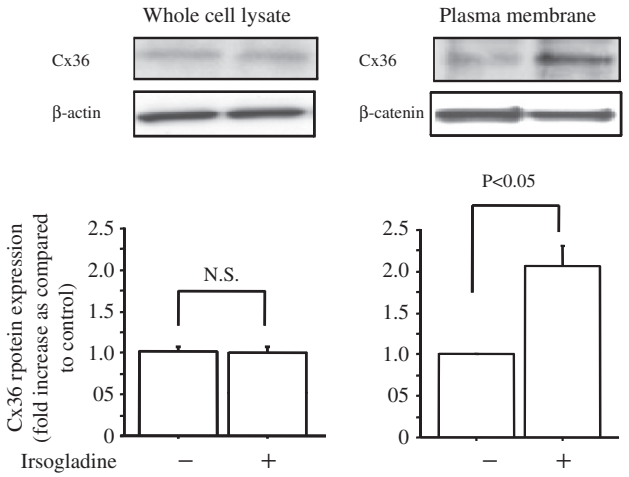

Fig. 3. Effect of irsogladine on Cx36 protein expression. MIN6 cells were incubated with or without 1.0×10^{-5} M irsogladine in KRB buffer for 30 min. Whole cell lysates or plasma membrane fractions were then prepared and were analyzed by immunoblotting using an anti-Cx36 antibody. Beta-actin or beta-catenin was used as a loading control of whole cell lysate or plasma membrane fraction, respectively. The intensity of the bands was quantified using Image-J software. Data are shown as means ± S.E.M. (n = 4).

4. Discussion

In the present study, we showed that irsogladine induced increased insulin secretion by MIN6 cells under high glucose conditions, whereas it had no effect on insulin secretion under low glucose conditions. Treatment with 18-beta-GRA inhibited the effect of irsogladine on insulin secretion under high glucose conditions. Furthermore, the level of the

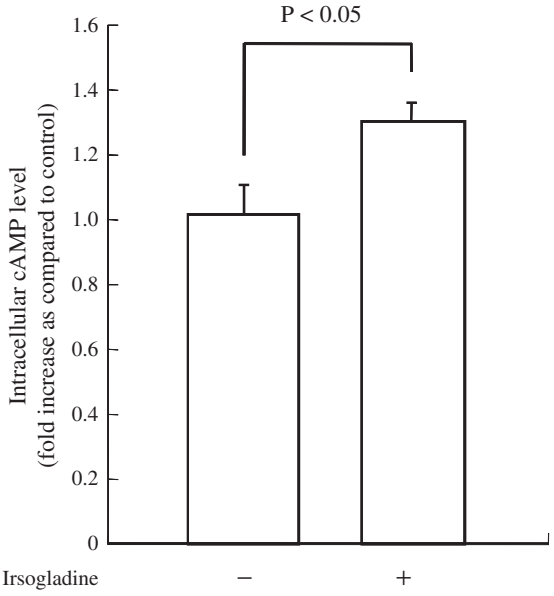

Fig. 4. Effect of irsogladine on intracellular cAMP levels. MIN6 cells were incubated with or without 1.0×10^{-5} M irsogladine in KRB buffer for 20 min following which intracellular cAMP levels were measured using a cAMP EIA kit. Data are shown as means ± S.E.M. (n = 3).

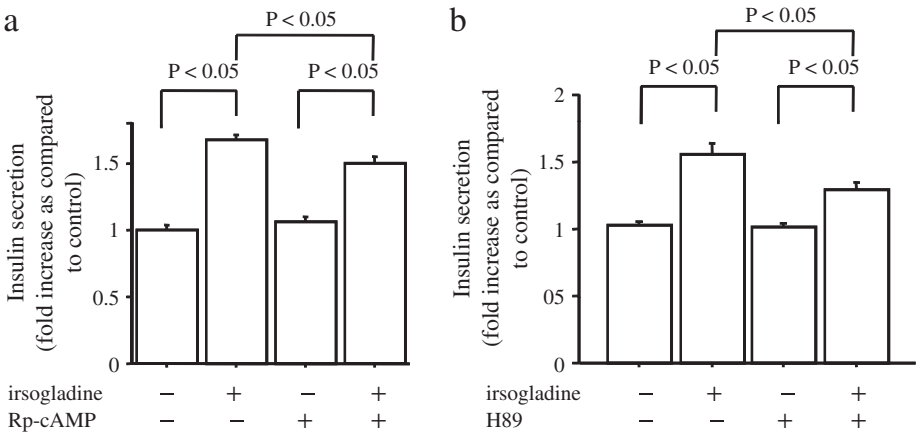

Fig. 5. Effect of a cAMP antagonist or a protein kinase A inhibitor on irsogladine-stimulated insulin secretion by MIN6 cells. MIN6 cells were incubated with or without 5.0×10^{-5} M Rp-cAMP for 30 min, followed by incubation with or without 1.0×10^{-5} M irsogladine and/or 5.0×10^{-5} M Rp-cAMP in KRB buffer for 30 min (a). MIN6 cells were incubated with or without 1.0×10^{-5} M irsogladine and/or 5.0×10^{-6} M H89 in KRB buffer for 30 min (b). The cells were then stimulated with 16.8 mM glucose for 60 min. Insulin concentration in the incubation media was measured using an ELISA. Data are shown as means ± S.E.M. (n = 4–6).

Cx36 protein in the plasma membrane fraction was increased by irsogladine treatment. These irsogladine treatments also resulted in a significant, but mild increase in intracellular cAMP levels. Moreover, treatment with a cAMP antagonist or a protein kinase A inhibitor partially inhibited irsogladine effects.

Normal insulin secretion requires the coordinated function of beta cells within pancreatic islets. Pancreatic beta cells are interconnected by gap junctions (Charollais et al., 2000; Kohen et al., 1983; MacDonald and Rorsman, 2006; Mears et al., 1995; Meda et al., 1990; Nlend et al., 2006; Ravier et al., 2005; Speier et al., 2007). The interaction of these channels across the extracellular space allows direct exchange of low molecular weight cytoplasmic molecules and ensures electrical coupling (Andreu et al., 1997; Charpantier et al., 2007; Kohen et al., 1979; MacDonald and Rorsman, 2006; Serre-Beinier et al., 2009). In the present study, we examined the effects of irsogladine, which is an agent reported to affect the gap junctions of other cell types, on insulin secretion by MIN6 cells. Irsogladine had no effect on insulin secretion under low glucose conditions. However, irsogladine induced increased insulin secretion by MIN6 cells under high glucose conditions. These data suggest that irsogladine is able to potentiate insulin secretion in MIN6 cells. The 18-beta-GRA that we used to inhibit gap junction channels has been successfully used for this purpose in previous studies (Davidson and Baumgarten, 1988; Yamamoto et al., 1998). Inhibition of the irsogladine effect on insulin secretion by 18-beta-GRA under high glucose conditions indicated that functional gap junctions are required for the effects of irsogladine on insulin secretion under high glucose conditions.

Gap junctions are specialized membrane regions consisting of hexametric assemblies of proteins (connexins) in the apposed membranes of adjacent cells (Laird, 2006) and have previously been shown to play a role in the regulation of insulin secretion (Nlend et al., 2006). Cx36 is the only connexin isoform that is expressed in beta cells and contributes to insulin secretion in the control of glucose levels and nutrient metabolism (Nlend et al., 2006; Serre-Beinier et al., 2000, 2009; Theis et al., 2004). Depletion of Cx36 causes decreased intercellular communication and inappropriate insulin secretion in beta cells (Ravier et al., 2005; Speier et al., 2007; Wellershaus et al., 2008). Cx36 is expressed in human islets and contributes to the exchange of specific molecules (Serre-Beinier et al., 2009). The mRNA expression of Cx36 correlates with insulin mRNA expression (Serre-Beinier et al., 2009). These data suggest that Cx36 is a native protein of human and mouse pancreatic islets, which mediates the coupling of insulin-producing beta cells, and contributes to the control of beta cell function. Pancreas perfusion studies using beta cell-specific Cx36 knockout mice showed impaired insulin secretion under physiologically high glucose conditions. However, insulin secretion increases under low glucose conditions in these mice, suggesting that gap junctions composed of Cx36 are required for proper insulin secretion under physiological glucose concentrations (Wellershaus et al., 2008). In the present study, irsogladine induced increased expression of the Cx36 protein in the plasma membrane fractions, suggesting that this increased expression of Cx36 in the plasma membrane may contribute to increased insulin secretion under high glucose conditions. Our data showed that irsogladine did not affect insulin secretion under low glucose conditions even though Cx36 expression in the plasma membrane was increased. It is possible that other pathways such as increased intracellular cAMP levels contributed to this result. In contrast, it has been reported that insulin secreting cells expressing a 10-fold higher level of Cx36 compared to control cells showed decreased secretion of insulin in response to increased glucose concentrations (Vozzi et al., 1995). Our data, but not the data of this previous study, suggest that gap junctions formed by increased Cx36 protein are involved in increased insulin secretion. The apparent discrepancies between the two studies may be caused by differences in the expression level of the Cx36 protein in each study. Several studies have shown that gap junctions suppress insulin secretion, especially at low glucose concentrations (Bavamian et al., 2007; Meda et al., 1990; Wellershaus et al., 2008) and contribute to appropriate insulin secretion at high glucose concentrations. Transgenic mice expressing a high amount of Cx32 in beta cells exhibit impaired insulin secretion (Bavamian et al., 2007). It is possible that overexpression of CX36 at a concentration more than ten folds that of endogenous CX36 causes suppression of insulin secretion that is too high to allow for appropriate insulin secretion. Adequate expression levels of Cx36 may be required for the regulation of appropriate insulin secretion. Irsogladine may adjust the level of Cx36 protein that is associated with the plasma membrane and thereby influence insulin secretion in response to different glucose concentrations. However, the role of Cx36 and gap junctions in insulin secretion is not yet fully understood, and further studies are necessary to clarify their functions.

Another reported effect of irsogladine is to increase intracellular cAMP levels (Ueda et al., 1991). It is well known that an increased level of intracellular cAMP stimulates insulin secretion under high glucose conditions in pancreatic islets and in insulin secreting cell lines, including MIN6 cells (Malaisse and Malaisse-Lagae, 1984; Prentki and Matschinsky, 1987; Seino et al., 2009). In our study irsogladine treatment resulted in a significant but mild increase in intracellular cAMP levels. In addition, inhibition of cAMP–protein kinase A pathway suppressed the effects of irsogladine. These data suggest that cAMP–

protein kinase A pathway was required for the effects of irsogladine. It has been reported that irsogladine-induced intracellular cAMP stimulates protein kinase A, resulting in the phosphorylation of specific connexins and causing translocation of those connexins to the plasma membrane (Kawasaki et al., 2002). It is possible that the increased cAMP that we observed following irsogladine treatment up-regulates membrane-associated Cx36 through protein kinase A activation. However, we were unable to evaluate the relationship between cAMP levels and Cx36 protein levels in the plasma membrane. The relationship between the intracellular cAMP level and connexins has not been fully elucidated. It is possible that irsogladine affects both Cx36 and intracellular cAMP levels and that the combination of these effects causes increased insulin secretion, especially under high glucose conditions. Further studies are required to elucidate the mechanism of the effects of irsogladine on insulin secretion.

In conclusion, we determined that irsogladine increases glucose-stimulated insulin secretion by MIN6 cells and that functional gap junctions and the cAMP–PKA system are required for this effect.

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