Loss of the voltage-gated proton channel Hv1 decreases insulin secretion and leads to hyperglycemia and glucose intolerance in mice

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Huimin Pang§, Xudong Wang§, Shiqun Zhao§, Wang Xi‡, Jili Lv†, Jiwei Qin†, Qing Zhao†, Yongzhe Che§, Liangyi Chen§½, and Shu Jie Li†‡

From the Department of Biophysics, School of Physics Science, Key Laboratory of Bioactive Materials, Ministry of Education, Nankai University, Tianjin 300071, China, the Institute of Molecular Medicine, Peking University, Beijing 100871, China, and the School of Medicine, Nankai University, Tianjin 300071, China

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Insulin secretion by pancreatic islet β-cells is regulated by glucose levels and is accompanied by proton generation. The voltage-gated proton channel Hv1 is present in pancreatic β-cells and extremely selective for protons. However, whether Hv1 is involved in insulin secretion is unclear. Here we demonstrate that Hv1 promotes insulin secretion of pancreatic β-cells and glucose homeostasis. Hv1-deficient mice displayed hyperglycemia and glucose intolerance because of reduced insulin secretion but retained normal peripheral insulin sensitivity. Moreover, Hv1 loss contributed much more to severe glucose intolerance as the mice got older. Islets of Hv1-deficient and heterozygous mice were markedly deficient in glucose- and K⁺-induced insulin secretion. In perfusion assays, Hv1 deletion dramatically reduced the first and second phase of glucose-stimulated insulin secretion. Islet insulin and proinsulin content was reduced, and histological analysis of pancreas slices revealed an accompanying modest reduction of β-cell mass in Hv1 knockout mice. EM observations also indicated a reduction in insulin granule size, but not granule number or granule docking, in Hv1-deficient mice. Mechanistically, Hv1 loss limited the capacity for glucose-induced membrane depolarization, accompanied by a reduced ability of glucose to raise Ca²⁺ levels in islets, as evidenced by decreased durations of individual calcium oscillations. Moreover, Hv1 expression was significantly reduced in pancreatic β-cells from streptozotocin-induced diabetic mice, indicating that Hv1 deficiency is associated with β-cell dysfunction and diabetes. We conclude that Hv1 regulates insulin secretion and glucose homeostasis through a mechanism that depends on intracellular Ca²⁺ levels and membrane depolarization.

Insulin secretion by pancreatic β-cells is precisely regulated by glucose homeostasis. Defective insulin secretion because of β-cell loss or dysfunction causes type 1 and type 2 diabetes (T2D), respectively (1). In the presence of high concentrations of glucose, β-cells metabolize glucose and generate ATP, which closes ATP-dependent K⁺ channels and results in membrane depolarization and the subsequently increased intracellular Ca²⁺ that triggers insulin granule release (1).

There are two different time stages in insulin secretion in humans (2) and rodents (3, 4), including a fast transient first phase and a slow sustained second phase. In patients with T2D, the first phase has almost disappeared, and the second phase is markedly decreased (5). Multiple factors influence the biphasic nature of GSIS, including intracellular Ca²⁺ levels, distinct pools of insulin granules, and metabolic signaling (6–8). A decrease in insulin secretion may be caused by a single- or multiple-step defect in the trafficking cycle of insulin granules, including granule biogenesis from the trans-Golgi network, subsequent maturation, recruitment to the plasma membrane, exocytosis, and endocytosis.

Glucose metabolism by pancreatic β-cells accompanies proton generation, which implies a mechanism of intracellular pH regulation behind insulin release stimulated by the sugar (9). Manipulating intracellular as well as extracellular pH could affect the insulin secretion process, which is associated with changes in membrane potential, ionic flux, and insulin release (9–11). Barg et al. (12) showed that the acidic pH in insulin granules might regulate priming of the granules for secretion, a process involving pairing of SNAREs on vesicles and target membranes to establish fusion competence. The voltage-gated proton channel Hv1 is extremely selective for protons and has no detectable permeability to other cations (13, 14). Hv1 is activated at depolarizing voltages and sensitive to the membrane pH gradient (13, 14). Hv1 sustains calcium entry in neutrophils and maintains intracellular alkalization to support the activity of human spermatozoa (15, 16). In our previous study, we identified that Hv1 is present in human and rodent pancreatic islet β-cells as well as β-cell lines (17). How-

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1 To whom correspondence may be addressed. E-mail: lychen@pku.edu.cn.

2 To whom correspondence may be addressed. Dept. of Biophysics, Key Laboratory of Bioactive Materials, Ministry of Education, Nankai University, 94 Weijin Rd., Nankai District, Tianjin 300071, China. Tel./Fax: 86-22-2350-6973; E-mail: shujiel@nankai.edu.cn.

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Figure 1. Mice deleted for Hv1 display hyperglycemia and impaired glucose tolerance because of reduced insulin secretion. A, body weight change of Hv1−/−, Hv1+/−, and WT mice (n = 24/genotype). Data are means ± S.D. B and C, basal blood glucose (B) and corresponding serum insulin (C) concentrations after fasting for 6 h in 4-month-old Hv1−/−, Hv1+/−, and WT mice (n = 13 for Hv1−/− and Hv1+/− and n = 10 for WT). Data are means ± S.D. ***, p < 0.001; Hv1−/− or Hv1+/− versus WT. D–F, HOMA parameters calculated from basal blood glucose (B) and corresponding serum insulin (C) concentrations. Data are means ± S.D., p < 0.05; **, p < 0.001; Hv1+/− or Hv1+/− versus WT. HOMA-IR was calculated as follows: fasting glucose (millimoles per liter) × fasting insulin (milliunits per liter) / 22.5. Homeostasis model assessment insulin sensitivity is the reciprocal of HOMA-IR. Homeostasis model assessment insulin secretion function was calculated as follows: 20 × fasting insulin (milliunits per liter) / (fasting glucose (millimoles per liter) – 3.6). G and H, Blood glucose levels measured in whole blood following i.p. injection of glucose (2 g/kg of body weight) in Hv1−/−, Hv1+/−, and WT mice (G) and AUC (H) (n = 12/genotype). Data are means ± S.E. ***, p < 0.001; Hv1+/− versus WT. I and J, insulin concentrations measured in sera of Hv1−/−, Hv1+/−, and WT mice following i.p. glucose challenge (2 g/kg of body weight) (I) and AUC (J) (n = 8/genotype). Data are means ± S.E. ***, p < 0.001; Hv1+/− or Hv1+/− versus WT. K, blood glucose levels measured in whole blood following i.p. insulin injection (1 unit/kg of body weight) in Hv1−/−, Hv1+/−, and WT mice (n = 12/genotype). Data are means ± S.E. *, p < 0.05; Hv1+/− versus WT.

ever, the regulatory mechanism of Hv1 for insulin secretion of pancreatic islet β-cells is not known.

In this study, we discovered a regulatory mechanism for Hv1 in the modulation of β-cell insulin secretory function. Our in vivo and in vitro studies demonstrated that Hv1-deficient mice exhibit hyperglycemia and glucose intolerance because of abnormally decreased insulin secretion. These data provide direct genetic evidence that Hv1 regulates biphasic insulin secretion and glucose homeostasis in mice, implying a potential link between Hv1 and the pathogenesis of diabetes mellitus.

Results

Hv1−deficient mice exhibit hyperglycemia and impaired glucose tolerance because of reduced insulin secretion

To assess the effect of Hv1 knockout on glucose homeostasis, glucose and insulin levels were measured in 4-month-old mice in a fasted state. The body weight curves of control (WT, Hv1+/+), heterozygous (Hv1+/−), and homozygous (KO, Hv1−/−) litter-mates were almost similar (Fig. 1A), but the blood glucose levels in the fasted state were markedly higher in heterozygous (9.45 ± 0.39 mmol/liter, n = 13, p < 0.001) and KO (9.75 ± 0.52 mmol/liter, n = 13, p < 0.001) mice compared with WT mice (6.3 ± 0.2 mmol/liter, n = 10) (Fig. 1B), whereas the fasting insulin levels in these three genotypes were not significantly different (Fig. 1C). To qualitatively estimate the effect of Hv1 on insulin resistance, insulin sensitivity, and β-cell function, we calculated Homeostasis model assessment insulin resistance (an index of insulin resistance), HOMA-β (an index of insulin secretory function), and HOMA-IS (an index of insulin sensitivity) from the corresponding glucose and insulin levels (18). The results showed that loss of Hv1 had a tendency for insulin resistance (Fig. 1D) because of decreased insulin sensitivity (Fig.
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The calculated results also showed that Hv1 deficiency significantly affected β-cell secretory function (Fig. 1E), which is involved in hyperglycemia.

To evaluate the impact of Hv1 on disposal of a glucose load, i.p. glucose tolerance tests (IPGTTs) were performed. Compared with WT mice, KO and heterozygous mice 4 months of age showed significantly higher glucose levels following an i.p. glucose load (2 g/kg of body weight) (Fig. 1, G and H). The corresponding serum insulin levels were significantly lower in Hv1-deficient mice throughout the IPGTT after glucose challenge compared with WT mice, providing evidence for an insulin secretion defect (Fig. 1E). The HOMA parameters calculated from basal blood glucose (A) and corresponding serum insulin (B) concentrations also showed no obvious insulin resistance in 2- and 6-month-old KO mice (Fig. 2, A and B). The fasting blood glucose levels in KO mice (9.7 ± 0.31 mmol/liter, n = 10, p < 0.001 for 2-month-old mice; 10.63 ± 0.47 mmol/liter, n = 10, p < 0.001 for 4-month-old mice) were also significantly higher than in WT mice (6.42 ± 0.27 mmol/liter, n = 10 for 2-month-old mice; 6.3 ± 0.2 mmol/liter, n = 10 for 4-month-old mice) (Fig. 2B). There was almost no difference in the blood insulin levels in the fasted state between 2- and 6-month-old KO mice (Fig. 2B). The HOMA parameters calculated from the corresponding blood glucose and insulin levels showed a potential tendency for insulin resistance in 2- and 6-month-old KO mice (Fig. 2, C and E) and significant dysfunction of β-cells (Fig. 2D). The IPGTT showed more serious glucose intolerance in 6-month-old KO mice than in 2-month-old KO mice (Fig. 2F and G), whereas WT mice showed no glucose intolerance at different ages. Although serum insulin levels in response to glucose in both 2- and 6-month-old KO mice were markedly lower than in WT mice (Fig. 2H), the IPITT showed no obvious insulin resistance in 2- and 6-month-old KO mice.

Hv1 deletion mice display an age-dependent development in glucose intolerance

To examine the age-dependent effect of Hv1 on glucose tolerance and insulin resistance, we performed IPGTTs and IPITTs in 2- and 6-month-old WT and KO mice as described above. The fasting blood glucose levels in KO mice (9.7 ± 0.31 mmol/liter, n = 10, p < 0.001 for 2-month-old mice; 10.63 ± 0.47 mmol/liter, n = 10, p < 0.001 for 4-month-old mice) were also significantly higher than in WT mice (6.42 ± 0.27 mmol/liter, n = 10 for 2-month-old mice; 6.3 ± 0.2 mmol/liter, n = 10 for 4-month-old mice) (Fig. 2A). There was almost no difference in the blood insulin levels in the fasted state between 2- and 6-month-old KO mice (Fig. 2B). The HOMA parameters calculated from the corresponding blood glucose and insulin levels showed a potential tendency for insulin resistance in 2- and 6-month-old KO mice (Fig. 2, C and E) and significant dysfunction of β-cells (Fig. 2D). The IPGTT showed more serious glucose intolerance in 6-month-old KO mice than in 2-month-old KO mice (Fig. 2F and G), whereas WT mice showed no glucose intolerance at different ages. Although serum insulin levels in response to glucose in both 2- and 6-month-old KO mice were markedly lower than in WT mice (Fig. 2H), the IPITT showed no obvious insulin resistance in 2- and 6-month-old KO mice.
KO mice (Fig. 2), indicating that hyperglycemia in Hv1-deficient mice is not caused by abnormal insulin sensitivity or T2D.

**Reduced insulin secretion of islets from Hv1-deficient mice**

To delineate the role of Hv1 in insulin secretion, we performed insulin secretion assays using isolated islets from WT, Hv1+/−, and KO mice. As shown in Fig. 3A, 16.7 mM glucose-induced insulin secretion was significantly reduced by 51% (n = 8, p < 0.001) and 78% (n = 8, p < 0.001) in Hv1+/− and KO islets compared with WT islets (n = 8). Basal insulin secretion (at 2.8 mM glucose) in Hv1+/− and KO islets was also significantly reduced by 60% (n = 8, p < 0.001) and 80% (n = 8, p < 0.001) compared with WT islets (n = 8). Direct depolarization elicited by an increase in extracellular K+ (60 mM KCl) also reduced insulin secretion by 59% (n = 8, p < 0.001) and 65% (n = 8, p < 0.001) in Hv1+/− and KO islets compared with WT islets (n = 8) (Fig. 3A), indicating that knockout of Hv1 prevents K+−induced insulin secretion in pancreatic β-cells. Together, these data indicate that loss of Hv1 in islets inhibits insulin secretion.

Next we examined Hv1 function in biphasic GSIS by static incubation and perfusion assays from pancreatic islets. In static incubation assays, we measured the amounts of first-phase (within the first 10 min) and second-phase (within the following 20 min) insulin secretion stimulated by 16.7 mM glucose in isolated islets in WT and KO mice. As shown in Fig. 3B, Hv1 deficiency strikingly impaired both first- and second-phase in GSIS. In an area under the curve (AUC) analysis (Fig. 7B), the AUC in KO islets was significantly decreased by 60% (p < 0.05) compared with that in the WT, which was consistent.
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Hv1 deficiency affects the size of insulin granules but not the number and docking of vesicles

Insulin is stored in large dense-core secretory granules in β-cells and released via granule exocytosis upon stimulation. The correct size, number, and docking to the cell membrane of insulin granules are necessary for insulin secretion in β-cells (19, 20). We used TEM to investigate whether loss of Hv1 disturbed vesicle distribution in β-cells. The ratios of small vesicles (<300 nm) and large vesicles (>400 nm) were increased by 4-fold and decreased by 47% in KO mice compared with WT mice, respectively, but the ratio of the medium vesicles (300–400 nm) showed no significant difference (Fig. 4, G and H). However, the total number of vesicles in β-cells in KO mice was the same as that in WT mice (Fig. 4I). This result might explain why there is a difference in insulin content between KO and WT mice (Fig. 3F). Docking of insulin granules plays an important role in regulating insulin secretion (20). A detailed quantitative electron microscopy analysis of β-cells was performed to study docked granules. The number of secretory granules close to (<100 nm) the cell membrane in β-cells in KO mice was similar to that in WT mice (Fig. 4J), indicating that loss of Hv1 does not affect granule docking in β-cells.

We further investigated the expression of syntaxin1A (Stx1a), vesicle-associated membrane protein 2 (VAMP2), and synaptotagmin-7 (Syt7), proteins related to insulin secretion, in isolated islets from WT and KO mice (21, 22). Their expression levels were slightly down-regulated, as determined by Western blotting (Fig. 4K). The reduced membrane fusion protein suggests that lack of Hv1 may affect fusion of insulin granules with the plasma membrane.

Hv1 deficiency affects glucose-induced Ca²⁺ oscillation and limits membrane depolarization

Glucose stimulates insulin secretion by induction of Ca²⁺-dependent electrical activity, which triggers exocytosis of insulin granules. Glucose-stimulated calcium (GSCa) signaling and GSIS show similar trajectories (23). Thus, GSCa can be used to assess the physiological response of islets to glucose stimulation. Ca²⁺ imaging is advantageous because it provides high temporal precision of real-time changes in response to stimuli at the level of the individual islet (24). The data presented in Fig. 5, A and B, are representative curves and average graphs of GSCa over time for islets isolated from WT and KO mice, respectively. The baseline, amplitude, timing, and duration of the biphasic response to glucose stimulation were calculated according to Fig. 5A. As shown in Fig. 5C, the mean value of cellular Ca²⁺ levels for baseline under basal conditions was increased by 12% in KO islets compared with the WT, suggesting that elevated [Ca²⁺], under low glucose does not lead to increased insulin release. After stimulation, both WT and KO islets displayed a large spike in calcium influx, and the mean values for peaks showed difference between KO and WT islets (Fig. 5D). However, the mean duration was decreased by 32% in KO islets compared with the WT (Fig. 5E), and the time to peak was decreased by 31% (Fig. 5F),
suggesting that loss of Hv1 results in dissociation between calcium signaling and GSIS.

In pancreatic β-cells, the increase in [Ca$^{2+}$], occurs with Ca$^{2+}$ entry across voltage-sensitive Ca$^{2+}$ channels activated by membrane depolarization (6). To confirm whether the change in calcium signaling by knockout of Hv1 is coupled to membrane polarization, the membrane potential difference was monitored with DiBAC4(3) fluorescence. As shown in Fig. 5, G and H, isolated islet β-cells from WT mice were depolarized significantly more than isolated islet β-cells from KO mice after glucose stimulation, indicating that Hv1 deficiency impairs glucose-induced membrane depolarization. Thus, the reduction in insulin secretion by Hv1 deficiency is involve in electrical activity and [Ca$^{2+}$] signaling.
Effect of Hv1 on cytosolic pH in pancreatic β-cells

To assess the effect of Hv1 on cytosolic pH in β-cells, cytosolic pH was evaluated by BCECF fluorescence. As shown in Fig. 6A, the pH in KO β-cells was lower about 0.1 than that in WT β-cells at both 2.8 and 16.7 mM glucose conditions. When we changed the glucose concentration from 2.8 to 16.7 mM, there was a slight synchronous reduction in pH in WT and KO β-cells (Fig. 6A). The pH under steady-state basal (2.8 mM) and high-glucose (16.7 mM) conditions in KO β-cells was also slightly lower compared with the corresponding WT β-cells, but there were no significant differences between them (Fig. 6B), indicating that knockout of Hv1 has no a significant effect on cytosolic pH in β-cells stimulated by glucose.

PMA is a well-known activator of Hv1 and has been used extensively for Hv1 function studies (25, 26). We measured PMA-induced insulin secretion in isolated islets from WT and KO mice. As shown in Fig. 6E, in the presence of 5 μM PMA, insulin secretion in isolated islets from WT mice increased 6.3-fold compared with the absence of PMA, whereas insulin secretion in KO islets was significantly decreased by 61% (p < 0.001) compared with WT islets.

To estimate the effect of PMA on cytosolic pH, the change in fluorescence intensity at 488 nm excitation was observed with addition of PMA solution. Interestingly, the fluorescence intensity for KO β-cells was significantly reduced compared with that of the WT (Fig. 6, C and D), indicating that PMA results in a decrease in cytosolic pH.

Hv1 is down-regulated in pancreatic β-cells in STZ-induced diabetic mice

To further determine the relationship between Hv1 and insulin secretion, WT and KO mice were treated with STZ to induce diabetes. We found that fasted blood glucose levels after STZ injection in KO mice were always higher than in WT mice (Fig. 7A), and the IPGTT also showed higher blood glucose levels in STZ-treated KO mice than in WT mice (Fig. 7B). These data show that STZ-treated KO mice seem to be more sensitive to diabetes induction.

The insulin content at 2.8 mM glucose was decreased by 91% in β-cells of STZ-treated WT mice (Fig. 7C), whereas insulin secretion stimulated by 16.7 mM glucose was reduced by 97% (Fig. 7D), which is a serious insulin deficiency. Immunohistochemical analyses of islets in control and STZ-treated WT mice using anti-insulin and anti-Hv1 antibodies also showed that the mean optical density of insulin- and Hv1-positive areas in STZ-treated WT mice were decreased by 53% and 60%, respectively, compared with control mice (Fig. 7E), demonstrating that the expression levels of insulin and Hv1 are significantly decreased in β-cells of STZ-treated diabetes mice, as shown in Fig. 7C, b and d. The lower expression of Hv1 in β-cells of diabetic mice...
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**Figure 6. Effects of glucose and PMA treatment on cytosolic pH and of Hv1 on PMA-stimulated insulin secretion.** A, effect of Hv1 on glucose-induced changes in cytosolic pH in isolated islet β-cells, determined by BCECF fluorescence. Glucose (final concentration, 16.7 mM) solution was added to the culture dishes without interruption of the recordings. B, cytosolic pH in β-cells isolated from WT and KO mice in the presence of 2.8 or 16.7 mM glucose (Glc). Data are means ± S.E. (n = 3/genotype). C, representative images of WT and KO islet β-cells loaded with BCECF dye in sodium-free solution to minimize the contribution of Na+/H+ exchange under basal conditions and in the presence of 5 μM PMA. Scale bar = 100 μm. D, change in fluorescence (F488/F0) with time, stimulated by PMA (final concentration, 5 μM). The PMA solution was added to the culture dishes without interruption of the recordings. E, PMA-induced insulin secretion from isolated islets from WT and KO mice (n = 8/genotype). Data are means ± S.E., *p < 0.05; ***, p < 0.001, versus corresponding WT; ###, p < 0.001 versus corresponding WT at 0 μM PMA.

**Discussion**

Here we show in vivo that Hv1 KO and heterozygous mice display hyperglycemia and glucose intolerance because of markedly decreased insulin secretion. In vitro, Hv1 deficiency causes a remarkable defect in glucose- and K⁺-induced insulin secretion, limiting the capacity for glucose-induced membrane depolarization that accompanies the reduced ability of glucose to raise the level of Ca²⁺ in islets, as evidenced by the decreased duration of individual calcium oscillations. These data indicate that Hv1 is required for insulin secretion and maintenance of glucose homeostasis and reveal a significant role of the proton channel in modulation of pancreatic β-cell function.

Hv1 is extremely selective for protons and has no detectable permeability to other cations (13, 14). Gating of Hv1 strongly depends on membrane depolarization and intracellular and extracellular pH (13–15, 26). The proton channel also sustains Ca²⁺ influx and enables neutrophils to generate calcium signals in response to chemoattractants, in addition to maintenance of normal cytosolic pH and membrane potential during the respiratory burst (15). Our previous work demonstrated that Hv1 mainly localizes to the insulin-containing granule membrane (17). Considering fusion of insulin-containing granules with the plasma membrane during exocytosis, Hv1 should also be in the plasma membrane in pancreatic islet β-cells. Therefore, Hv1 might be involved in regulating insulin-containing granules, cytosolic pH, membrane potential, and Ca²⁺ influx in pancreatic β-cells.

Manipulation of extracellular and intracellular pH in pancreatic islets or β-cells is associated with changes in membrane potential, ionic flux, and insulin release (9–11). Glucose-induced changes in cytosolic pH in islet β-cells are coupled to glucose metabolism and associated with triggering insulin release (10, 11). Glucose-induced priming of insulin secretion, which is thought to be mediated by the amplifying pathway (28), has been proposed to be linked to pH changes in β-cells (29). It has been suggested for decades that the proton gradients in insulin-containing granules might be involved in fusion of secretory vesicles to the target membrane (30, 31), which might affect triggering of vesicles for secretion, a process involving pairing of SNARE proteins on vesicles and target membranes to establish fusion competence (12). Dependence of insulin secretion on Hv1 activity reflects that insulin-containing granules and cytosolic pH are directly involved in secretion of secretory granules. A change in membrane potential in Hv1-deficient pancreatic β-cells under glucose stimulation condition also reflects the pH dependence of the secretory machinery as well as altered sensitivity of the secretory machinery to [Ca²⁺]ᵢ.

The fact that heterozygous mice also have hyperglycemia with a low insulin level illustrates that Hv1 is at an important control point in the metabolic pathway regulating insulin secretion and that relatively small changes in Hv1 activity are likely to have important effects on insulin secretion. Similar effects...
have been observed for glucokinase (32). Hv1 expression levels are significantly decreased in β-cells of STZ-treated diabetes mice and mice with chronic hyperglycemia—induced dysfunction. These data further confirm that Hv1 is closely related to insulin secretion. The findings of this study clearly demonstrate that Hv1 plays an important role in positively regulating GSIS.

β-Cell failure is associated with not only decreased β-cell insulin secretory function but also reduced overall β-cell mass (33). In this study, there was no difference in islet morphology between KO and WT mice and only a very modest decrease in islet size and β-cell mass. The smaller size of Hv1-deficient pancreatic islets may be related to the decrease in β-cell mass and result from impaired insulin secretion function. It is important to note that, in vitro, siRNA-mediated knockdown of Hv1 in isolated islets and INS-1 (832/13) cells caused decreased GSIS (17), suggesting that the in vivo decrease in insulin secretion in KO mice was not due to an in vivo β-cell developmental defect.

Glucose metabolism generates ATP and closes ATP-regulated K+ channels, leading to membrane depolarization. Membrane depolarization of β-cells leads to activation of voltage-sensitive L-type Ca2+ channels with a subsequent rise in intracellular Ca2+, which then drives vesicular exocytosis (34). Sulfonylureas, such as tolbutamide and glibenclamide, bind to the SUR subunit of ATP-sensitive K+ channels and induce channel closure (35). Knockout of Hv1 inhibits tolbutamide-
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Figure 8. Proposed operation of the voltage-gated proton channel Hv1 on signal transduction pathways of insulin secretion stimulation.

Glucose

\[
\downarrow cAMP \leftrightarrow ATP \downarrow Tol \downarrow PKA \downarrow K_{\text{ATP}} \downarrow PMA
\]

Ca\(^{2+}\) Influx

Depolarization \leftrightarrow PKC \downarrow VDCC (L-type)

Hv1

Insulin Secretion

closure of K\(_{\text{ATP}}\) channels. PMA, as an Hv1 activator, activates PKC (26) and opens Hv1, resulting insulin secretion. The important defect that ablates the Hv1 gene could be predicted from the reduced depolarization in β-cells and the reduced ability of glucose to raise the level of Ca\(^{2+}\) in islets, as we observed in this work. Without activation of Hv1, depolarization by closure of the K\(_{\text{ATP}}\) channel might be weakened a lot, and these distal effects could not occur.

Blood glucose control is a complex metabolic process involving numerous tissues and organs. Global knockout of Hv1 might affect glucoregulation in many tissues and organs. Insulin is the only hypoglycemic hormone secreted from pancreatic β-cells. Hv1 is highly expressed in pancreatic β-cells (17). Knockout of Hv1 remarkably reduces glucose-stimulated insulin secretion in isolated islets and serum insulin levels in mice, suggesting that hyperglycemia and glucose intolerance in Hv1KO mice are due to decreased insulin secretion in pancreatic β-cells. The decreased insulin and Hv1 expression in pancreatic islets in diabetes model mice imply a link of Hv1 with insulin secretion and diabetes. Taken together, the data in this study demonstrate that Hv1-deficient mice exhibit hyperglycemia and impaired glucose tolerance because of reduced insulin secretion. This finding provides direct evidence of a functional role of the proton channel in maintenance of glucose homeostasis. This study describes a novel pathway regulating β-cell secretory function in which Hv1 sustains entry of calcium ions into β-cells by regulating membrane depolarization to promote increased insulin secretion responses.

Experimental procedures

Animals and treatments

Mice bearing a targeted disruption in the VSOP/Hv1 (VSOP/Hv1\(^{-/-}\)), backcrossed eight times, kindly provided by Dr. Y. Okamura, School of Medicine, Osaka University), as described previously (37). WT mice (VSOP/Hv1\(^{+/+}\)) were of the same genetic background (C57BL/6J). Animals were kept in a pathogen-free facility under a 12-h light/dark cycle with access to water and a standard mouse diet (Lillico Biotechnology). Genotyping was performed by PCR as described by Ramsey et al. (25). Experiments were performed with 2- to 4-month-old male mice unless indicated otherwise. All animal husbandry and experiments were approved by and performed in accordance with guidelines from the Animal Research Committee of Nankai University.

Blood glucose and insulin determination

Blood glucose levels were measured from blood obtained from the tail vein after fasting for 6 h using an automated glucometer (One Touch, Johnson & Johnson). For glucose tolerance tests, mice 4 months of age were fasted for 6 h before i.p. injection of glucose (2 g/kg of body weight), and blood glucose concentration was measured 0, 15, 30, 60, and 120 min after injection. For insulin tolerance tests, mice were fasted for 6 h before intraperitoneal injection with insulin (1.0 units/kg of body weight), and blood glucose concentration was measured at 0, 15, 30, 60, and 120 min.

For serum insulin measurements, glucose (2 g/kg of body weight) was injected i.p. Venous blood was collected at 0, 2, 5,
Isolation of pancreatic islets

Pancreatic islets were isolated according to the collagenase digestion method described by Lacy and Kostianovsky (38), with slight modifications. Krebs–Ringer bicarbonate HEPES (KRBH) buffer (135 mM NaCl, 3.6 mM KCl, 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂, and 10 mM HEPES (pH 7.4)) was used for islet isolation. Isolated islets were cultured overnight in RPMI 1640 medium (Gibco) containing 10% FBS in a humified 5% CO₂ atmosphere at 37 °C before hand-picking for experiments as described previously (17).

Insulin secretion assay

Insulin secretion from isolated islets was measured as described previously (17). Briefly, after overnight culture, the islets were aliquoted into microtubes (20 size-matched islets/tube) and preincubated for 1 h in KRBH-BSA buffer containing 2.8 mM glucose at 37 °C. Then the islets were incubated with different stimuli in KRBH-BSA buffer for 1.5 h. To measure biphasic insulin secretion, islets were first stabilized for 30 min in KRBH-BSA buffer containing 2.8 mM glucose at 37 °C. Following establishment of a baseline with 2.8 mM glucose in KRBH-BSA buffer for 10 min, islets were picked by hand and plated into KRBH-BSA buffer containing 16.7 mM glucose for 10 min and then in another dish for 20 min. Insulin levels in fractions were measured. Islet perifusion was performed at 10 min and then in another dish for 20 min. Insulin levels in fractions were measured. Islet perifusion was performed at 37 °C using a system designed in-house. Forty equal size islets were paired between WT and KO mice in each experiment. Islets were perifused with KRBH-BSA buffer. The flow rate was controlled by a perfusion pump at 500 μl/min, and fractions were collected in microtubes for insulin determination. Total insulin and proinsulin content in isolated islets was extracted with acidic ethanol and determined using rat/mouse proinsulin and mouse and rat insulin ELISA (Mercodia) according to the manufacturer’s protocol.

Immunohistochemistry

Immunohistochemistry was performed as described previously (17).

Transmission EM (TEM)

Islets isolated from six 4-month-old animals were fixed in 2.5% glutaraldehyde for ultrathin (70 nm) sectioning and imaging by TEM (Hitachi HT7700). For quantification of vesicles, images were captured using TEM at magnification ×10,000–15,000. Docked vesicles were counted, with vesicles whose outer surface was within 100 nm of the plasma membrane considered docked granules. At least 50 random sections were used to capture microscopic fields from six mice per group. Then about 50 cells of each genotype were analyzed before identifying their genotype.

Western blotting

Western blotting was performed with anti-α-tubulin (Developmental Studies Hybridoma Bank), anti-VAMP2 (Protein-tech), anti-Stx1A (Synaptic Systems), and anti-Syt7 (Synaptic Systems), with final dilutions of 1:1000, 1:500, 1:1000, and 1:250, respectively, as described earlier (39). The intensity of individual bands was quantified using ImageJ software. Each experiment was performed in triplicate.

Measurements of intracellular Ca²⁺

Isolated islets were incubated in KRBH buffer with basal glucose containing 2 μM Fura-2/AM dye (Invitrogen) for 30 min at 37 °C. The glass coverslips were placed on the stage of an inverted microscope (Eclipse Ti, Nikon). Epifluorescence mode with a ×10 objective was used, stepping to 340 nm excitation and 380 nm excitation for 100 ms every 3 s. Regions of interest were outlined and monitored simultaneously during the whole experimental procedure using MetaFluor software. The resultant fluorescent signals of Fura-2 were successfully monitored at a wavelength of 535 nm with the help of a digital CMOS camera (Zyla 4.2 Plus, Undor). Individual images and intensity values were recorded along with the emission data. After data acquisition, an area of the coverslip without cells was measured as background. Background fluorescence was then subtracted, and the ratio of emission with 340 nm excitation to emission with 380 nm excitation was calculated, showing the F340/F380 ratio for real-time intensity measurements.

Measurements of membrane potential

The voltage-sensitive bisoxonol fluorescent dye DiBAC₄(3) was used to study the membrane potential changes of dispersed islet β-cells. Freshly isolated mouse islets were dispersed at room temperature into single cells by addition of 0.1% trypsin-EDTA solution for 2 min with gentle pipetting. The dispersed cells were washed with RPMI 1640 medium containing 10% FBS and placed on 0.01% poly-1-lysine–precoated glass coverslips for 12 h before experiments. The cells were incubated with 1 μM DiBAC₄(3) for 30 min at 37 °C in basal glucose in KRBH buffer prior to fluorescence measurement. Data were expressed as an average of three experiments (80–100 cells/experiment).

Measurements of cytosolic pH

Cytosolic pH (pHᵣ) was assessed using BCECF/AM (Invitrogen) as described previously (40). β-Cells seeded on glass coverslips were loaded with 2 μg/ml of BCECF-AM (Molecular Probes) at 37 °C for 30 min, inserted in a thermostatic chamber, and imaged through a ×10 objective. To determine the cytosolic pH, fluorescence at excitation wavelengths of 436 and 495 nm was recorded at an emission wavelength of 535 nm. Calibration of fluorescence versus pH was performed by equilibration of external and internal pH with nigericin (10 μM) in a high K⁺ buffer with a pH range of 5.5 to 8.0. High-K⁺ buffer contained 145 mM KCl, 2.8 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, and 20 mM HEPES (or MES). The relative fluorescence ratio values were plotted against corresponding pHᵣ values, which allowed determination of the unknown pHᵣ. The data were expressed as an average of three experiments (50–100 cells/experiment). To observe the change in cytosolic pH induced by PMA (an activator of Hv1), fluorescence at an excitation wavelength of 488 nm was recorded at an emission wavelength of 535 nm. Analysis was performed with MetaMorph software. To exclude any contribution of the Na⁺/H⁺ antiporter, experi-
Loss of Hv1 inhibits insulin secretion

The pancreatic islet β-cell line INS-1 (832/13) was obtained from Dr. Hans Hohmeier (Duke University) and grown in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 2 mM glutamate, 1 mM sodium pyruvate, and 55 μM β-mercaptoethanol in a humidified 5% CO₂ atmosphere at 37 °C.

siRNA silencing

To down-regulate Hv1 expression level in INS-1 (832/13) cells, siRNA targeting the Hv1 gene (5’-CTCAACAGAAATGG-GAGAAT-3’) and a scramble sense sequence (5’-TTCTCCG-CTACAAGAAATGG-3’) were used as previously described (17).

Statistical analysis

All statistics were performed using SPSS20.0 software. Comparison of the mean between groups was performed by t test. p < 0.05 was considered significant.

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