Functional characterization of hyperpolarization-activated cyclic nucleotide-gated channels in rat pancreatic β cells

Yi Zhang¹*, Yunfeng Liu¹*, Jihong Qu², Alexandre Hardy¹, Nina Zhang¹,³, Jingyu Diao¹, Paul J Strijbos⁴, Robert Tsushima¹, Richard B Robinson², Herbert Y Gaisano⁴, Qinghua Wang¹,³ and Michael B Wheeler¹

¹Departments of Physiology and Medicine, University of Toronto, Room 7310, Medical Sciences Building, 1 King’s College Circle, Toronto, Ontario, Canada M5S 1A8
²Department of Pharmacology, Columbia University, New York, New York 10032, USA
³Division of Endocrinology and Metabolism, Li Ka-Shing Knowledge Institute, St Michael’s Hospital, Room 7005, Queen Wing, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8
⁴Neurology and Gl Centre of Excellence for Drug Discovery, GlaxoSmithKline, New Frontiers Science Park, Harlow CM19 5AW, UK

(Correspondence should be addressed to Y Zhang; Email: zany.zhang@utoronto.ca; Q Wang; Email: qinghua.wang@utoronto.ca)

*Y Zhang and Y Liu contributed equally to this work

Abstract

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels regulate pacemaker activity in some cardiac cells and neurons. In the present study, we have identified the presence of HCN channels in pancreatic β-cells. We then examined the functional characterization of these channels in β-cells via modulating HCN channel activity genetically and pharmacologically. Voltage-clamp experiments showed that over-expression of HCN2 in rat β-cells significantly increased HCN current (Ih), whereas expression of dominant-negative HCN2 (HCN2-AYA) completely suppressed endogenous Ih. Compared to control β-cells, over-expression of Ih increased insulin secretion at 2.8 mmol/l glucose. However, suppression of Ih did not affect insulin secretion at both 2.8 and 11.1 mmol/l glucose. Current-clamp measurements revealed that HCN2 over-expression significantly reduced β-cell membrane input resistance (Rin), and resulted in a less-hyperpolarizing membrane response to the currents injected into the cell. Conversely, dominant negative HCN2-AYA expression led to a substantial increase of Rin, which was associated with a more hyperpolarizing membrane response to the currents injected. Remarkably, under low extracellular potassium conditions (2.5 mmol/l K+), suppression of Ih resulted in increased membrane hyperpolarization and decreased insulin secretion. We conclude that Ih in β-cells possess the potential to modulate β-cell membrane potential and insulin secretion under hypokalemic conditions.

Journal of Endocrinology (2009) 203, 45–53

Introduction

The current produced by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, termed Ih, has been recorded in a variety of cardiac cells and neurons. Unlike most voltage-dependent channels, HCN channels are activated by membrane hyperpolarization and are permeable to both Na+ and K+ ions. To date, four mammalian HCN isoforms (HCN1–4) have been cloned. A major function of Ih is to serve as a pacemaker current in some excitable cells (DiFrancesco 1993, Robinson & Siegelbaum 2003). This pacemaker function is believed to play important roles in regulating heart rate and spontaneous electrical activity of neurons. In pyramidal neuron dendrites (Poolos et al. 2002), amacrine cells (Koizumi et al. 2004) and thalamocortical relay neurons (Meuth et al. 2006), Ih has been proposed to participate in controlling and stabilizing resting membrane potential. Ih has also been thought to be involved in the responses to sour taste, neuronal plasticity, and dendritic integration (reviewed in Pape (1996), Robinson & Siegelbaum (2003) and Wahl-Schott & Biel (2009)).

Pancreatic β-cells are electrically excitable cells that secrete insulin to maintain blood glucose homeostasis. A number of ion channels contribute to this function. Among these channels, the ATP-sensitive K+ (KATP) channel initiates membrane depolarization at high glucose and the voltage-dependent Ca2+ channel plays a key role for action potential firing and insulin secretion (Rorsman et al. 1994). We had reported that voltage-dependent K+ (Kv) channels are involved in the repolarization phase of the action potential; and that blockade of the Kv channel prolongs the action potential duration and enhances insulin secretion (MacDonald et al. 2002, MacDonald & Wheeler 2003). Recently, we demonstrated the presence of an HCN-encoded Ih in the pancreatic β-cell (El Kholy et al. 2007). In the present study, we further examine its functional characterization in regulation of electrical activity of pancreatic β-cells.
Materials and Methods

Islet isolation and cell culture
Islets of Langerhans were isolated from male Wistar rats weighing 250–350 g by collagenase digestion and separated by density gradient centrifugation, as described previously (MacDonald et al. 2001). Animal procedures were performed in accordance with the University of Toronto’s Animal Care Committee’s ethical guidelines. To obtain single islet cells, the intact rat islets were dispersed in dispase II solution (Roche) at 37 °C for 5 min and the single cells were plated on glass coverslips. Intact islets or dispersed islet cells were cultured in RPMI 1640 medium containing 11·1 mmol/l glucose supplemented with 10% fetal bovine serum, 10 mmol/l HEPES, 100 units/ml penicillin, and 100 μg/ml streptomycin for 24–72 h before experiments. Under these conditions, the infection efficiency was routinely 80–90% as determined by GFP expression.

Western blotting analysis
Expression of HCN2 proteins in rat islets adenovirally infected were determined by western blot. Rat brain was used as a positive control. Cell lysates (25 μg) were subjected to SDS-PAGE and transferred to polyvinylidene difluoride–plus membranes (Fisher Scientific Ltd, Nepean, ON, Canada). Membranes were probed with the antibody for HCN2 (1:800 dilution). The bound primary antibody was detected with peroxidase-conjugated secondary anti-rabbit antibody (1:30 000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and visualized by chemiluminescence (ECL-Plus, GE Healthcare, Mississauga, ON, Canada).

Electrophysiology
Cells were patch-clamped in perforated whole-cell configuration at 33–34 °C. β-Cells were identified by cell size (> 4 pF) and their depolarization response to 11·1 mmol/l glucose (Wendt et al. 2004, Manning Fox et al. 2006). The measurements were performed using EPC-9 amplifier and PULSE software from HEKA (Lambrecht, Germany). Patch pipettes were pulled from 1·5 mm thin-walled borosilicate glass tubes using a two-stage Narishige micropipette puller (Narishige Co., Tokyo, Japan). The pipettes had typical resistances of 3–6 MΩ when fire polished and filled with an intracellular solution containing 140 mmol/l KCl; 1 mmol/l MgCl2; 0·05 mmol/l EGTA; 10 mmol/l NaCl; 10 mmol/l HEPES; pH 7·3 adjusted with KOH. Gramicidin was added to the intracellular solution to a final concentration of 60 μg/ml immediately before the experiment. Standard extracellular solutions contained 138 mmol/l NaCl; 5·6 mmol/l KCl; 1·2 mmol/l MgCl2; 2·6 mmol/l CaCl2; 5 mmol/l HEPES; 11·1 mmol/l glucose; pH 7·4 adjusted with NaOH. For membrane potential recordings in Fig. 1, 2·8 mmol/l glucose was also used. For the low potassium extracellular solution used in Fig. 2, 138 mmol/l NaCl and 5·6 mmol/l KCl from standard extracellular solution were replaced by 140 mmol/l NaCl and 2·5 mmol/l KCl. All perforated patch recordings were achieved with serial resistance below 25 MΩ, and leak subtraction protocol was not applied. In current-clamp mode, membrane potential responses were measured from whole rat islets. The membrane input resistance (Rm) was estimated by Ohm’s law Rm = U/I, where U is the membrane voltage measured at the end of current (I) injection. The equilibrium potential for potassium (E钾) was calculated by Nernst equation. ZD7288 was purchased from Tocris (Ellisville, MO, USA).
Static insulin secretion assay

After 72 h post-infection, the islets (10/vial) were pre-incubated with glucose free Krebs–Ringer HEPES buffer (KRB, 125 mmol/l NaCl, 5.6 mmol/l KCl, 1.28 mmol/l CaCl₂, 5.0 mmol/l NaCO₃, 25 mmol/l HEPES, and 0.1% (w/v) BSA) for 30 min, followed by incubation with 2.8 or 11.1 mmol/l glucose in KRB for 90 min. The supernatants of the islets were collected and centrifuged at 300 g for 10 min to remove cell debris and insulin was measured by RIA (Dai et al. 2006). The islets were then used for cell viability assessment. The insulin secretion data were normalized by the islet cell viability via an XTT absorbance assay. The XTT cell proliferation assay (Proliferation Kit II; Roche Applied Sciences) was performed according to the manufacturer’s instructions and as we have previously shown (Targonsky et al. 2006). Briefly, after insulin secretion experiments, the islets were immediately incubated with the XTT reagent for 6 h and the absorbance readings at 490 nm were recorded.

Islet perifusion secretory assay

After 72 h post-infection, batches of 50 islets were placed in perifusion chambers with a capacity of 1.3 ml at 37°C and perifused with KRB at a flow rate of 1 ml/min as described above. For the low potassium extracellular solution used in Fig. 3, 128 mmol/l NaCl and 2.5 mmol/l KCl were used to replace the original concentrations of NaCl and KCl in the KRB. Islets were stimulated with 2.8 or 16.7 mmol/l glucose in the presence or absence of Forskolin (10 μM, Sigma–Aldrich Ltd) plus 3-isobutyl-1-methylxanthine (IBMX; 100 μM, Sigma–Aldrich Ltd) as indicated. Fractions were collected for insulin determination using a RIA kit (Linco Research, St Louis, MO, USA). At the end of each perifusion, islets were collected and lysed in acid ethanol for assessment of insulin content. Results are presented as insulin secreted normalized to total insulin content.

Statistical analysis

All data are presented as means ± S.E.M. Statistical analysis was done by Student’s t-test or paired t-test and significance was assumed at a P value of <0.05. All calculations were made by SigmaPlot (SigmaPlot 2001, SPSS Science, Chicago, IL, USA). Patch clamp data were analysed with IGOR Pro3.12 software (Wavemetrics, Lake Oswego, OR, USA).

Results

HCN channels are expressed in rat β-cells

Four members of the Hcn gene family (Hcn1–4) are currently known (Santoro et al. 1997, 1998, Ludwig et al. 1998).

Figure 1 Influence of Iₕ on membrane potential in standard extracellular solution containing 5-6 mmol/l KCl: membrane potentials were recorded without current injection in rat islet β-cells transduced with (A) GFP or (B) HCN2-AYA in the presence of 11.1 mmol/l (11.1 G) and 2.8 mmol/l (2.8 G) glucose. (C) Summary of mean membrane potentials at 11.1 and 2.8 mmol/l glucose (n = 5 for each).

Figure 2 Influence of Iₕ on membrane hyperpolarization in extracellular solution containing 2-5 mmol/l KCl: membrane potentials were recorded without current injection. (A) Rat islet β-cells were perfused with solution containing 11.1 mmol/l glucose and were then challenged with 200 μM diazoxide (Diaz) in the absence or presence of 50 μM ZD7288 (ZD) followed by perfusion of 300 μM tolbutamide (Tolb). (B) Bar graph shows the normalized changes of mean membrane potentials (to Δ₁ induced by diazoxide), Δ₂ is the excessive change induced by ZD7288 at the presence of diazoxide (n = 6). Data shown are mean ± S.E.M., *P < 0.05 by paired t-test.

Figure 3 Influence of Iₕ on insulin secretion in extracellular solution containing 2-5 mmol/l KCl: (A) islet perifusion insulin secretion assay was performed and the amount of insulin were measured at 2-8 mmol/l glucose (2-8 G) and 16-7 mmol/l glucose (16-7 G) as indicated, insulin released was normalized to total insulin content. (B) Average of area under curves (AUC) of insulin levels during islet perifusion from A (n = 4). The first phase (Ph1) of insulin secretion at 16-7 mmol/l glucose was quantified from 10 to 21 min and the second phase (Ph2) from 21 to 50 min from A. Data shown are mean ± S.E.M., *P < 0.05 compared with GFP control.

Statistical analysis

All data are presented as means ± S.E.M. Statistical analysis was done by Student’s t-test or paired t-test and significance was assumed at a P value of <0.05. All calculations were made by SigmaPlot (SigmaPlot 2001, SPSS Science, Chicago, IL, USA). Patch clamp data were analysed with IGOR Pro3.12 software (Wavemetrics, Lake Oswego, OR, USA).

Results

HCN channels are expressed in rat β-cells

Four members of the Hcn gene family (Hcn1–4) are currently known (Santoro et al. 1997, 1998, Ludwig et al. 1998).
We therefore used specific antibodies of each of these HCN isoforms, which detected the presence of all four HCN isoforms in primary rat β-cells (Fig. 4). These results are consistent with our previously reported real-time PCR analysis of rat islet mRNA (El Kholy et al. 2007). Of note, some non-β-cells (see arrows pointing at non-insulin staining cells) are also positive for HCN, indicating that HCN channels are also expressed in other types of islet cells (Zhang et al. 2008).

Electrophysiological properties of native $I_h$ in rat β-cells

We investigated the native $I_h$ in rat β-cells. As shown in Fig. 5A, hyperpolarizing voltages elicited slow activating inward currents (Fig. 5Aii), while application of HCN channel blocker, ZD7288 (50 μM), inhibited these currents (Fig. 5Aiii). On average, ZD7288 blocked $67.9 \pm 7.2\%$ ($P<0.01$, $n=6$) of the sustained $I_h$ obtained at $-140$ mV. Since both Na$^+$ and K$^+$ permeate HCN channels, the reversal potentials of $I_h$ are between approximately $-50$ and $-20$ mV in many native pacemaker cell types (Pape 1996, Accili et al. 2002). To test the ion selectivity of $I_h$ in rat β-cells, a current–voltage ($I/V$) relationship of the fully activated HCN channel was plotted and the reversal potential of $I_h$ was obtained at $-38$ mV (Fig. 5Bii and Biii), suggesting that $I_h$ in β-cells is a mixed cationic current of Na$^+$ and K$^+$ (Tian & Shipston 2000). These data clearly showed that native $I_h$ in rat β-cells possesses

Figure 4 Expression of HCN proteins in pancreatic rat islet cells: representative confocal laser scanning microscopy images of dispersed rat islet cells, which were dual-stained for each of the HCN isoforms (red) and insulin (green). Light (left) and merged images (right) are shown in the lower panels of each group.
250 ms test pulses ranging from K(66 I activated HCN channels. To obtain the (B) Determination of current–voltage relationship of the fully b reversal potential is K of ZD7288 on I Yu et al 67.9 dependent sixteenfold greater than that in control cells at current density in HCN2-transduced cells was approximately with GFP control (Fig. 6Bi). Figure 6C illustrates the voltage-over-expression of HCN2 in the islet b significant increase in inward currents (Fig. 6Biii) compared in infection (Fig. 6 A). Patch-clamp study demonstrates that channel gene expression and current magnitude in primary an adenoviral gene transfer strategy to manipulate HCN on endogenous nant-negative HCN2-AYA almost completely suppressed currents against test potentials (right panel, n=6) showing the level of HCN2 proteins was effectively enhanced after transplantation. The tail currents displayed typical sigmoidal properties (Ludwig Sumino 1998). As shown in Fig. 6 D, the tail currents induced by a K pulse were measured and the data were fit using the Boltzmann equation (Yagi & Sumino 1998). As shown in Fig. 6D, the tail current amplitudes displayed typical sigmoidal properties (Ludwig et al. 1999). The half-maximal activation (V1/2) voltage was −97 ± 1 mV in GFP control and −96 ± 1 mV in HCN2 group (Fig. 6D).

Influence of I\textsubscript{h} on insulin secretion

Since ion channels play a pivotal role in regulation of insulin secretion, we next sought to determine whether insulin secretion is affected by HCN channel modulation. Our static insulin secretion data show HCN2-over-expression increased insulin secretion at 2.8 mmol/l glucose, but not change the secretion at 11.1 mmol/l glucose when compared with GFP control (Fig. 7A and B). No difference was observed for insulin secretion between HCN2-AYA-transduced and GFP-transduced islets at both glucose concentrations (Fig. 7A and B). To further confirm the results, we employed islet perfusion to investigate the effect of HCN channels on dynamic insulin secretion. We find HCN2-AYA did not change the insulin secretion pattern compared to GFP control even in the presence of forskolin/IBMX (Fig. 7C and D).

Effects of over-expression or dominant-negative suppression of HCN channels on I\textsubscript{h}

In order to investigate the functional role of I\textsubscript{h}, we employed an adenoviral gene transfer strategy to manipulate HCN channel gene expression and current magnitude in primary β-cells (Qu et al. 2001). Cells transduced with GFP alone were used as control. Western blotting results show that the level of HCN2 proteins was effectively enhanced after infection (Fig. 6A). Patch-clamp study demonstrates that over-expression of HCN2 in the islet β-cells resulted in a significant increase in inward currents (Fig. 6Bi) compared with GFP control (Fig. 6Bi). Figure 6C illustrates the voltage-dependent I/V relationships of the HCN channel. The mean current density in HCN2-transduced cells was approximately sixteenfold greater than that in control cells at −140 mV (66 ± 9 pA/pF for HCN2, n=7; 4±1 ± 0.5 pA/pF for GFP control, n=6; P<0.001); in contrast, expression of dominant-negative HCN2-AYA almost completely suppressed endogenous I\textsubscript{h} (0±3 ± 0.3 pA/pF for HCN2-AYA, n=7; P<0.001 versus control, Fig. 6Bi and C).

To determine the steady-state activation of these channels, tail currents induced by a −140 mV pulse were measured and the data were fit using the Boltzmann equation (Yagi & Sumino 1998). As shown in Fig. 6D, the tail current amplitudes displayed typical sigmoidal properties (Ludwig et al. 1999). The half-maximal activation (V1/2) were obtained as −97 ± 1 mV in GFP control (n=6) and −96 ± 1 mV in HCN2 (n=6), respectively. Data shown are mean ± S.E.M., *P<0.05, **P<0.01 and ***P<0.001, respectively, compared with the control.
During islet perifusion from C (content. (D) Average of area under curves (AUC) of insulin levels for forskolin plus IBMX. Insulin released was normalized to total insulin content (Fig. 8 Ai–iv), the difference between voltages induced by −20 and −140 pA injected currents (ΔV = V−20 pA − V−140 pA) was calculated. We found HCN2 over-expression significantly decreased ΔV compared to GFP control (26 ± 4 mV for HCN2 overexpression, n = 7; 67 ± 6 mV for GFP control, n = 6; P < 0.001, Fig. 8C), suggesting that Ih can prevent membrane potential from hyperpolarization in β-cells.

**Influence of Ih on membrane hyperpolarization**

We therefore next examined whether HCN channel modulation can affect the natural membrane potential responses. Membrane potential recordings from whole rat islets were performed in standard extracellular solution containing 5-6 mmol/l KCl. Figure 1 shows membrane potential responses at 11.1 and 2-8 mmol/l glucose concentrations in GFP- (Fig. 1A) or HCN2-AYA-transduced islets (Fig. 1B). It is shown that dominant-negative HCN2-AYA did not cause significant change of the mean membrane potentials at low or high glucose concentrations compared with GFP control (−41 ± 3 mV at 11.1 mmol/l glucose and −61 ± 2 mV at 2.8 mmol/l glucose).

**Influence of Ih on membrane input resistance (Rin)**

Membrane input resistance (Rin) is an important parameter of the intrinsic membrane properties, which determines membrane voltage responses to input currents (i.e. the currents flowing across the membrane). In general, the membrane voltage response is more sensitive to input current when Rin is high. It has been shown that Ih participates in regulation of Rin and thereby cell excitability in neurons (Magee 1998, Poolos 2002, 2006). To assess if Ih influences Rin in rat β-cells, we measured voltage responses to a set of injected hyperpolarizing currents under current-clamp mode. As shown in GFP-transduced cells, hyperpolarizing current produced a depolarizing voltage sag (or inflexion) (Fig. 8 Ai–iv, indicated by dashed arrow); this voltage sag reflected the activation of Ih that depolarizes the membrane potential. Compared to GFP control, the hyperpolarizing current resulted in a more significant depolarizing sag in HCN2-transduced cells because of the enhanced Ih (Fig. 8 Ai–iv). Figure 8B shows that the mean Rin (calculated from −20 to −60 pA of injected currents) was lower in HCN2 (from 935 ± 115 MΩ to 548 ± 54 MΩ, n = 7, P < 0.05 versus control) than in GFP control cells (from 1375 ± 159 to 998 ± 88 MΩ, n = 8), in contrast, the mean Rin of HCN2-AYA ranged from 2179 ± 306 to 1540 ± 100 MΩ being substantially higher (n = 6, P < 0.05 versus control). Since Ih has been reported to limit membrane potential from increased hyperpolarization in neurons (Poolos et al. 2002), we thus tested if this occurs in rat β-cells. As seen (Fig. 8 Ai–iv), the difference between voltages induced by −20 and −140 pA injected currents (ΔV = V−20 pA − V−140 pA) was calculated. We found HCN2 over-expression significantly decreased ΔV compared to GFP control (26 ± 4 mV for HCN2 overexpression, n = 7; 67 ± 6 mV for GFP control, n = 6; P < 0.001, Fig. 8C), suggesting that Ih can prevent membrane potential from hyperpolarization in β-cells.

**Figure 7** Influence of Ih on insulin secretion: (A and B) the islet static insulin secretion assay was performed and the amount of insulin were measured at 2.8 mmol/l glucose (2.8 G) and 11.1 mmol/l glucose (11.1 G), data were then normalized to the absorbance from XTT assay (n = 5). (C) The islet perfusion insulin secretion assay was performed and the amount of insulin were measured at 2.8 mmol/l glucose (2.8 G) and 16.7 mmol/l glucose (16.7 G) in the presence or absence of forskolin (10 μM) plus IBMX (100 μM). Data are mean ± S.E.M., *P < 0.05 versus control.

**Figure 8** Influence of Ih on input resistance (Rin): (Ai) stimulus protocol under current-clamp mode, resting potential was held at −40 mV by injecting constant current. The voltage responses were induced by additional hyperpolarizing currents ranging from −20 to −140 pA with a 20 pA increment. Representative current-clamp recordings in response to injected hyperpolarizing currents in rat β-cells transduced with GFP (Aii), HCN2 (Aiii) or HCN2-AYA (Aiv) are shown. For clarity, only voltages induced by −20, −60 and −140 pA injected currents are included. The dashed arrow indicated depolarizing ‘sag’. (B) Summary of input resistance obtained by the average of the voltage responded to a set of injected hyperpolarizing currents (K). (C) Group data for the difference between the two voltages in response to −20 and −140 pA of injected currents (ΔV = V−20 pA − V−140 pA) showing the stabilizing effect of Ih on membrane potential. Data shown are means ± S.E.M., *P < 0.05 and **P < 0.001, respectively, compared with the control.

**Journal of Endocrinology** (2009) 203, 45–53

www.endocrinology-journals.org

Downloaded from Bioscientifica.com at 12/27/2018 06:23:07AM via free access
2.8 mmol/l glucose for HCN2-AYA, \( n=5 \); vs \(-41 \pm 2 \text{ mV} \) at 11.1 mmol/l glucose and \(-55 \pm 4 \text{ mV} \) at 2.8 mmol/l glucose for GFP control, \( n=5 \), \( P>0.05 \); Fig. 1C).

We then tested the membrane potential responses from non-adeno-virus-infected islets in extracellular solution containing low potassium (2.5 mmol/l KCl) and 11.1 mmol/l glucose. As shown in Fig. 2, \( K_{\text{ATP}} \) channel opener diazoxide (200 \( \mu \text{M} \)) evoked remarkable hyperpolarization (\(-77 \pm 5 \text{ mV} \)), application of ZD7288 (50 \( \mu \text{M} \)) caused further hyperpolarization (\(-84 \pm 4 \text{ mV} \)) which could be reversed by \( K_{\text{ATP}} \) channel blocker, tolbutamide (300 \( \mu \text{M} \)). In average, ZD7288 caused 17% increased hyperpolarization compared to the membrane potential change induced by diazoxide (Fig. 2B).

**Influence of \( I_h \) on insulin secretion in extracellular solution containing 2.5 mmol/l KCl**

Using islet perfusion, we examined biphasic glucose-stimulated insulin secretion in islets at low potassium conditions. At 2.8 mmol/l glucose, no difference was observed between HCN2-AYA and GFP groups (3.6 \( \pm \) 0.5 vs 3.7 \( \pm \) 0.2, Fig. 3) as quantified by area under the curve analysis. At 16.7 mmol/l glucose, islets treated with HCN2-AYA displayed a similar level of insulin secretion in the first phase, and a markedly lower level in the second phase compared with GFP control (52.6 \( \pm \) 1.9 vs 63.7 \( \pm \) 3.2, Fig. 3).

**Discussion**

We have recently demonstrated the presence of HCN channels in mouse and rat pancreatic \( \beta \)-cells (El Kholy et al. 2007), whereas their functions are still unclear. We and others found that the HCN channel antagonist, ZD7288, has a \( I_h \)-independent inhibitory effect on exocytosis, and the three other HCN channel antagonists, caesium, clobradine, zatebradine lead to suppression of Kv channels (Paolisso et al. 1985, Satoh & Yamada 2002, Gonzalez-Iglesias et al. 2006, El Kholy et al. 2007). These \( I_h \)-independent effects hampered use of these reagents in some functional studies. To circumvent these problems, in the present study, we investigated the functional characterization of HCN channels by manipulating HCN channel expression genetically in primary \( \beta \)-cells.

Our voltage-clamp data clearly show that over-expression of HCN2 can effectively enhance \( I_h \), and over-expression of HCN2-AYA almost completely suppresses \( I_h \). It has been proposed that different HCN isoforms could co-assemble to form heteromers of HCN channels (Chen et al. 2001, Whitaker et al. 2007). Some evidence from studies using dominant-negative strategies has confirmed this notion. For example, in the neonatal ventricle, HCN2-AYA almost completely suppressed the native current mainly encoded by HCN2 and HCN4 in a dominant-negative manner (Er et al. 2003). It is also reported that a dominant-negative HCN2 pore mutant was able to suppress both HCN2 and HCN1 wild-type channels (Xue et al. 2002). Here, the fact that HCN2-AYA almost completely blocked native \( I_h \) in rat \( \beta \)-cells (where four HCN isoforms are present), indicates different HCN isoforms can co-assemble to form heteromeric channel complexes in pancreatic \( \beta \)-cells.

In the current-clamp experiments, our results reveal that suppression of \( I_h \) significantly enhanced membrane input resistance (\( R_{\text{in}} \)), while over-expression of \( I_h \) reduced \( R_{\text{in}} \), indicating that \( I_h \) is a regulator in determining \( R_{\text{in}} \) in pancreatic \( \beta \)-cells. \( R_{\text{in}} \) is an important parameter of the intrinsic membrane properties. According to Ohm’s law (\( V=I R_{\text{in}} \)), modulation of \( R_{\text{in}} \) will adjust the response of cell membrane potential (\( V \)) to the input current stimulus (\( I \)). Thus, our finding suggests that \( I_h \) may play a role to modulate \( \beta \)-cell membrane potential responses by modulating \( R_{\text{in}} \). Of note, besides the traditional voltage- and time-dependent component of HCN current, various studies suggest there also is an instantaneous, or voltage-independent, component (\( I_{\text{inst}} \)) of HCN currents that may represent ion flow through a ‘leaky’ channel (Proenza et al. 2002, Proenza & Yellen 2006). Therefore, both components may contribute to the effect of HCN channels on \( R_{\text{in}} \), and this may explain the observation that there were differences in input resistance between groups during injection of \(-20 \text{ pA} \) current (Fig. 8B), where the corresponding voltage is positive to the threshold for activation of the time-dependent component of HCN currents (around \(-70 \) to \(-80 \text{ mV} \)).

HCN current is reported to function as a safety inward current that helps prevent the membrane from hyperpolarization so that the cell membrane potential is maintained at appropriate levels (Williams et al. 2002). However, our data show that suppression of \( I_h \) did not significantly affect membrane potential at either low or high glucose in standard extracellular solution containing 5-6 mmol/l KCl (Fig. 1), indicating the native \( I_h \) may not be sufficient to influence the islet cell membrane potential at the conditions tested. We then examined the effect of \( I_h \) on membrane hyperpolarization in a low potassium extracellular solution ([K\(^+\)]\(_o\)) because theoretically, decrease of the [K\(^+\)]\(_o\) could shift the cell resting potential to a more hyperpolarized voltage, where HCN channels are in a more activated state. Indeed, under low [K\(^+\)]\(_o\) conditions, blockade of \( I_h \) by ZD7288 induced an increased membrane hyperpolarization. The effect of ZD7288 on membrane potential was unlikely due to effects on other channels, because we find that ZD7288 has no effects on voltage-dependent potassium channels, calcium channels and \( K_{\text{ATP}} \) channels (El Kholy 2007). The results therefore indicate that \( I_h \) in \( \beta \)-cells possess the potential to counteract membrane over-hyperpolarization at least under low [K\(^+\)]\(_o\) conditions.

Our previous report suggests that knock-down of HCN channel protein by siRNA does not influence insulin secretion under normal K\(^+\) conditions in insulinoma MIN6 cells (El Kholy et al. 2007). In the present study, we...
have verified the results by dominant-negative suppression of HCN channels using HCN2-AYA in rat islets. Our data demonstrate suppression of \( I_h \) did not affect insulin secretion and application of forskolin/IBMX to increase intracellular cAMP could not change the influence of \( I_h \) on insulin secretion at standard extracellular K\(^+\) concentrations (Fig. 7), suggesting \( I_h \) is not sufficient to modulate insulin release under normal \([K^+]_o\) conditions.

Under low \([K^+]_o\) conditions, however, a higher level of insulin secretion (especially for the second phase) was observed in control group at 16.7 mmol/l glucose but not at 2-8 mmol/l glucose in comparison with HCN2-AYA (Fig. 3), suggesting that HCN channels can facilitate glucose-induced insulin secretion under the conditions tested. This phenomenon is probably due to the activation of HCN channels rendered by the preceding low glucose, low \([K^+]_o\) conditions (where HCN channels in \(\beta\)-cells are in a more activated state compared with normal \([K^+]_o\) conditions), because studies have revealed that activation of HCN channels can also lead to enhancement of subsequent stimulus-induced synaptic transmission in dorsal root ganglion neurons (Yu et al. 2004) and crayfish (Beaumont & Zucker 2000, Beaumont et al. 2002). The mechanism of HCN channel modulation of exocytosis remains to be elucidated, but two possibilities have been raised: 1) fractional Ca\(^{2+}\) influx and accumulation through HCN channels (Yu et al. 2004), 2) the involvement of actin cytoskeleton in the interaction between HCN channels and exocytotic machinery (Beaumont & Zucker 2000, Beaumont et al. 2002, Zhong & Zucker 2004). Further studies are needed to clarify the involved mechanisms. Nevertheless, in the case of \( I_h \) in \(\beta\)-cells, our results suggest a potential role for HCN channels in regulating insulin secretion under low \([K^+]_o\) conditions.

It is worth pointing out that hypokalemia (i.e. serum K\(^+\) concentration <3-6 mmol/l) is thought to be the most common electrolyte abnormality encountered in clinical practice, and is found in over 20% of hospitalized patients (Gennari 1998). In diabetes mellitus, hypokalemia often develops, particularly in patients with diabetic ketoacidosis (Gennari 1998). Thus, it is likely that \( I_h \) in \(\beta\)-cells may play a protective role under these pathophysiological conditions.

Acknowledgements

The authors thank C J Mahowald and Y C Huang (University of Toronto, Canada) for assistance with manuscript preparation.

References


El Kholy W 2007 The role of hyperpolarization activated cyclic nucleotide modulated and voltage gated potassium channels in pancreatic beta-cell function. *University of Toronto*.


Received in final form 29 July 2009
Accepted 4 August 2009
Made available online as an Accepted Preprint 4 August 2009