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Presence of functional hyperpolarisation-activated cyclic nucleotide-gated channels in clonal alpha cell lines and rat islet alpha cells

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Abstract

Aims/hypothesis The hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels, discovered initially in cardiac and neuronal cells, mediate the inward pacemaker current (I_f or I_h). Recently, we have demonstrated the presence of HCN channels in pancreatic beta cells. Here, we aim to examine the presence and function of HCN channels in glucagon-secreting alpha cells.

Methods RT-PCR and immunocytochemistry were used to examine the presence of HCN channels in alpha cells. Whole-cell patch-clamp, calcium imaging and glucagon secretion experiments were performed to explore the function of HCN channels in alpha cells.

Results HCN transcripts and proteins were detected in alpha-TC6 cells and dispersed rat alpha cells. Patch-clamp recording showed hyperpolarisation-activated currents in alpha-TC6 cells, which could be blocked by HCN channel inhibitor ZD7288. Glucagon secretion RIA studies demonstrated that at both low and high glucose concentrations (2 and 20 mmol/l), ZD7288 significantly enhanced glucagon secretion in alpha-TC6 and IN-R1-G9 cell lines. Conversely, activation of HCN channels by lamotrigine significantly suppressed glucagon secretion at the low glucose concentration. Calcium imaging studies showed that blockade of HCN channels by ZD7288 significantly increased intracellular calcium in alpha-TC6 cells, while lamotrigine or the Na^+ channel blocker tetrodotoxin suppressed the effect of ZD7288 on intracellular calcium. Furthermore, we found the HCN channel inhibitors ZD7288 and cilobradine both significantly increased glucagon secretion from rat islets.

Conclusions/interpretation These results suggest a potential role for HCN channels in regulation of glucagon secretion via modulating Ca^{2+} and Na^+ channel activities.

Keywords Alpha cell · Glucagon secretion · HCN channel · Pancreatic islet

Abbreviations

$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
GABA	γ-aminobutyric acid
HCN channel	hyperpolarisation-activated cyclic nucleotide-gated channel
I/V	current–voltage relationship
K_{ATP} channel	ATP-sensitive K^+ channel
KRB	Krebs–Ringer bicarbonate
TTX	tetrodotoxin
$V_{1/2}$	membrane potential of half-maximal activation

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Introduction

Homeostasis of blood glucose is controlled mainly by insulin and its opposing hormone glucagon. High blood

glucose stimulates insulin secretion from pancreatic beta cells but suppresses glucagon secretion from pancreatic alpha cells. It is known that glucose-induced insulin secretion occurs via the glucose-sensing pathway, which involves the closure of ATP-sensitive K^+ channels (K_{ATP} channels) with resultant membrane depolarisation, and the activation of voltage-gated L-type Ca^{2+} channels and subsequent Ca^{2+}-dependent exocytosis of insulin from the beta cells [1]. However, the mechanism regulating glucagon secretion remains relatively unclear.

Insulin is known as a physiological suppressor of glucagon secretion [2–4]. There is evidence that substances including Zn^{2+} [5], γ-aminobutyric acid (GABA) [6–8] released from beta cells and somatostatin secreted from neighbouring delta cells [9] have important roles in the modulation of glucagon secretion. Thus, it has been proposed that glucose may induce suppression of glucagon release by indirect actions via a paracrine mechanism. Recent studies have suggested that the mechanisms underlying glucagon regulation may also involve a direct effect of glucose on alpha cell stimulus–secretion coupling, in which ion channels play an important role [10, 11].

It is well established that electrical activity in beta cells mainly depends on activity of K_{ATP} channels, L-type Ca^{2+} channels and delayed rectifying K^+ channels [1, 12, 13]. Unlike in beta cells, studies using rodent, human and guinea pig pancreatic alpha cells have suggested that more Ca^{2+} channel subfamilies, i.e. T- and N-type Ca^{2+} channels and tetrodotoxin (TTX)-sensitive Na^+ channels are also important in the regulation of alpha cell electrical activity [11, 13–17]. One important feature of these alpha cell ion channels is their voltage-dependent inactivation when the cell membrane is depolarised [14, 17, 18]. Accordingly, a model for glucagon secretion has been proposed that highlights the important roles of ion channels in regulating glucagon secretion. In this model, action potential firing (and glucagon secretion) in alpha cells occurs only within a narrow window of the membrane potentials that allows opening of voltage-dependent Na^+ channels and T- or N-type Ca^{2+} channels. At low glucose conditions, intermediate K_{ATP} channel activity maintains alpha cell membrane potential at the activation threshold for Na^+ and T- and N-type Ca^{2+} channels, causing a rise in cytosolic Ca^{2+} concentration and triggering glucagon secretion [10, 11, 13, 14, 16]. However, when the membrane potential is depolarised (e.g. K_{ATP} channels close under high glucose conditions), this will voltage-inactivate the Na^+ and T- and N-type Ca^{2+} channels, resulting in the cessation of action potential firing and suppression of glucagon secretion [10, 11, 14, 18]. On the other hand, when the alpha cell membrane potential is excessively hyperpolarised (e.g. a large fraction of K_{ATP} channels opening under diazoxide treatment), the Na^+ and Ca^{2+} channels will switch to a closed state, thus silencing the alpha cell regenerative electrical activity and inhibiting glucagon secretion [11]. These studies suggest that the coordination of different ion channels in alpha cells composes the fine regulatory mechanism that controls glucagon secretion. Here, we report that a new ion channel, the hyperpolarisation-activated cyclic nucleotide-gated (HCN) channel, may play a role in the control of glucagon secretion in alpha cells.

HCN channels are members of a superfamily of voltage-gated cation channels that are permeable to both Na^+ and K^+ ions and produce a slowly activating inward current. Unlike most voltage-dependent channels, HCN channels are activated by membrane hyperpolarisation. HCN channels were initially identified in cardiac and neuronal cells, and play a key role in regulation of heart rate and spontaneous electrical activity of neurons [19–22]. To date, four mammalian HCN isoforms (HCN1–4) have been cloned. Studies on isoform distribution showed that while HCN3 is found primarily in neurons [23, 24], the other three isoforms are found in heart and brain [23, 25]. The HCN transcripts are also detected in several other organs, including skeletal muscle and lung [24]. Recently, we had discovered that HCN channels are present in pancreatic beta cells [26]. In the present study, we examined the presence and function of HCN channels in pancreatic alpha cells.

Methods

Islet isolation and cell culture Pancreatic islets were isolated from 250–350 g male Wistar rats (Charles River, Quebec, QC, Canada) by collagenase digestion and separated by density gradient centrifugation, as described previously [26]. Intact islets were cultured in RPMI 1640 medium containing 11.1 mmol/l glucose supplemented with 10% (vol./vol.) fetal bovine serum, 10 mmol/l HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin for 24 h before experiments. Alpha-TC6 cells (a kind gift from Y. Moriyama, Okayama University, Okayama, Japan) and IN-R1-G9 cells (a kind gift from P. Brubaker, University of Toronto, Canada) were maintained in DMEM (Gibco Invitrogen, Burlington, ON, Canada), containing fetal bovine serum (10% vol./vol.), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulphate and 55 mg/500 ml sodium pyruvate, at 37°C in an atmosphere of humidified air (95%) and CO_2 (5%). In studies involving serum starvation, serum was replaced by 0.1% (wt/vol.) BSA in DMEM and incubated with the cells for 1 h. All animal procedures were approved by the Animal Care Committees at St Michael's Hospital and the University of Toronto.

RT-PCR Total cellular RNA was extracted from cells using Trizol (Invitrogen) following the manufacturer's instruc-

tions. HCN mRNA transcripts were amplified using a one-step RT-PCR kit (Qiagen, Valencia, CA, USA). Briefly, 100 ng total RNA was used in 25 μl one-step RT-PCR reactions containing 0.4 mmol/l dNTPs and 0.6 μmol/l of each primer. Water was used to replace the template for the negative control. PCR conditions were 50°C for 30 min, and 95°C for 15 min, and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min followed by a 10 min extension at 72°C. The products were separated on a 1% (wt/vol.) agarose gel and visualised with ethidium bromide. The HCN gene-specific primers used were as follows: HCN1, sense 5′-CAGCATGTCTGACCTCTGGA-3′, antisense 5′-TATCTT CTGGCGCATGTCAG-3′; HCN2, sense 5′-ATGTCCTTC CACAAACTGCC-3′, antisense 5′-TCCCCTCTCGGATGA TGTA-3′; HCN3, sense 5′-GTGGAAATCGAGCAGGA GAG-3′, antisense 5′-GCAGGAAGAAAGTGTCCGAG-3′; and HCN4, sense 5′-GCGTTTTGAGGTCTTTCAGC-3′, antisense 5′-TCATGGGATACTCCTCCAGC-3′.

Immunostaining Alpha-TC6 cells and rat islets were dual-stained for glucagon and HCN channels using a protocol similar to one used in a previous report [27]. In brief, cells or islets grown on coverslips were fixed with paraformal-dehyde (3.7% [wt/vol.] in PBS) and permeabilised with 0.2% (vol./vol.) Triton X-100. After blocking with PBS containing 5% (vol./vol.) goat serum and 1% (wt/vol.) BSA for 60 min, an antibody to HCN2 (1:100; Alamone Labs, Jerusalem, Israel) was added and left overnight at 4°C. After washing out excessive primary antibodies, cells or islets were incubated with FITC-conjugated goat anti-rabbit IgG (1:500; Jackson Immuno Research Laboratories, West Grove, PA, USA) for 30 min. The second staining was performed using an antibody to glucagon (1:500; Dako, Carpinteria, CA, USA) and a Cy3-conjugated secondary antibody (1:500; Jackson Immuno Research) as described above. The images were visualised using a Leica TCS 4D laser confocal fluorescence microscope (Leica, TCS-NT, Microsystems, Bensheim, Germany).

Electrophysiology Cells were patch-clamped in the conventional whole-cell configuration at room temperature. The measurements were performed using an EPC-9 amplifier and PULSE software from HEKA Electronik (Lambrecht, Germany). Patch pipettes were pulled from 1.5 mm thin-walled borosilicate glass tubes using a two-stage Narishige micropipette puller (Tokyo, Japan) and had typical resistances of 3–6 MΩ when fire polished and filled with an intracellular solution containing (mmol/l) 130 KCl; 10 NaCl; 0.5 $MgCl_2$; 1 EGTA; 5 HEPES; 5 MgATP; pH 7.3 with KOH. Extracellular solutions contained (mmol/l) 110 NaCl; 0.5 $MgCl_2$; 1.8 $CaCl_2$; 5 HEPES; 30 KCl; 10 glucose; pH 7.4 with NaOH. ZD7288 was obtained from Tocris (Ellisville, MO, USA).

Calcium imaging Experiments were carried out using an Olympus BX51W1 fluorescent microscope (Olympus Canada, Markham, ON, Canada). Changes in intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were assessed using Fura-2 AM. Alpha-TC6 cells on coverslips were loaded with 2.5 μmol/l Fura-2 AM for 45 min in incubation buffer at 37°C and 5% CO_2–95% air. Then coverslips were transferred to an open chamber on the microscope stage for imaging and perfused at 1 ml/min. The incubation and perfusion buffer contained (mmol/l): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 10 HEPES, pH 7.4. To depolarise the cells, 30 mmol/l NaCl was replaced with 30 mmol/l KCl. Cells were excited by dual excitation at 340/380 nm and emission was detected by a 510 nm band pass filter using a 415 nm beam splitter. Experiments were performed at 36–37°C using a TC-324B Heater Controller (Warner Instruments, Hamden, CT, USA).

Glucagon secretion Glucagon secretion in the clonal alpha cells was measured using a glucagon RIA kit (Linco Research, St Charles, MO, USA) as described previously [27]. For islet assay, Krebs–Ringer bicarbonate buffer (KRB) was used containing (mmol/l) 115 NaCl, 5 KCl, 24 $NaHCO_3$, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 2% (wt/vol.) BSA. Islets were pre-incubated at 37°C for 30 min in KRB supplemented with 5.6 mmol/l glucose and then transferred to 1.5 ml tubes for assay (five islets per sample). The islets were first incubated in 500 μl KRB with 11 mmol/l glucose for 1 h at 37°C, and then in 500 μl KRB with 1 mmol/l glucose for an additional 1 h at 37°C. The supernatant fractions (450 μl) from each sample were taken at the end of the incubation period and stored at −20°C until assayed for glucagon. The islet pellets were lysed with acid ethanol (0.09 mol/l HCl in 75% [vol./vol.] ethanol) for DNA quantification; glucagon secretion data were normalised by total DNA of each sample.

Statistical analysis All data are presented as means±SEM. Statistical analysis was done by a Student's *t* test or paired *t* test or ANOVA using $n-1$ custom hypotheses tests as appropriate. Patch-clamp data were analysed with IGOR Pro3.12 software (Wavemetrics, Lake Oswego, OR, USA) and the data of fluorescent measurements were processed using PSI-PLOT (Poly Software International, Salt Lake City, UT, USA). Significance was assumed at $p < 0.05$.

Results

HCN channels are expressed in glucagon-secreting alpha cells RT-PCR showed the presence of the transcripts coding for HCN proteins in glucagon-secreting alpha-TC6 cells and rat islets (Fig. 1a). Immunostaining predominantly
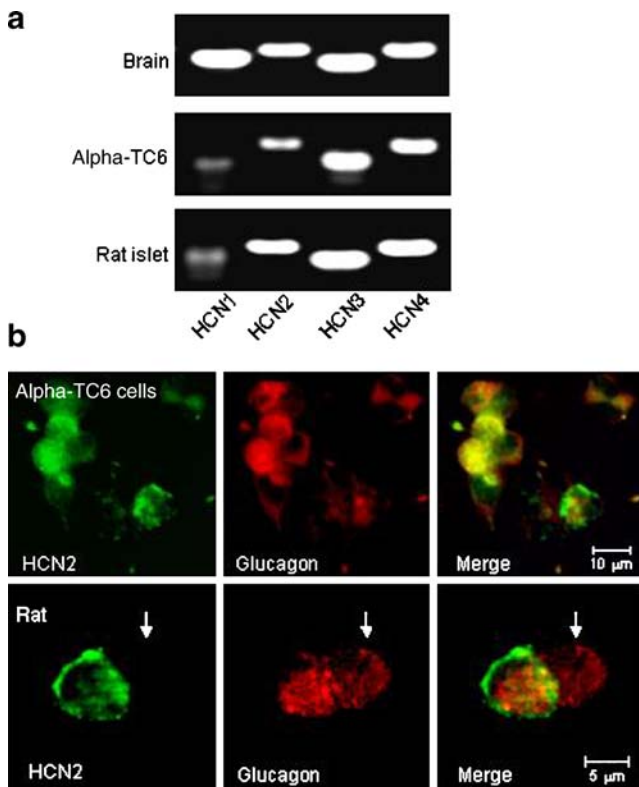
Fig. 1 Presence of HCN transcripts and proteins in alpha cells. **a** Total RNA from alpha-TC6 cells and rat islets was reverse transcribed into cDNA and PCR analysis was performed with specific primers for the HCN1–4 subtypes. In the negative control reaction, the template was omitted (not shown). **b** Confocal immunocytochemistry of alpha-TC6 cells and dispersed rat islet cells using the antibody against the HCN2 subtype. Scale bar, 10 μm. The white arrows indicate the rat islet cell that is glucagon-positive but not HCN2. In the negative control staining, either the primary antibodies were omitted or COS-7 cells were used (not shown). The data shown represent $n=3$ or 4 independent experiments

detected HCN2 proteins in alpha-TC6 cells and dispersed rat alpha cells (Fig. 1b). In the control experiments, no positive staining was observed when omitting the primary antibodies during the staining procedures (not shown). Interestingly, we also found that while most glucagon-positive alpha cells are HCN-positive, a few glucagon-positive cells were not associated with HCN protein (Fig. 1b [white arrows indicate the glucagon-positive but HCN2-negative rat islet cell]), which is also suggestive of a specific immunostaining.

Electrophysiological properties of HCN channels in alpha-TC6 cells Cells were clamped from a holding potential of −40 mV to various hyperpolarising conditioning voltages (−140 to −50 mV in 10 mV increments) for 3 s followed by a step to −140 mV (Fig. 2a). The recordings showed that the hyperpolarising voltages induced inward currents with slow activation kinetics (Fig. 2b). To construct the steady-state activation curve of these channels, the tail currents
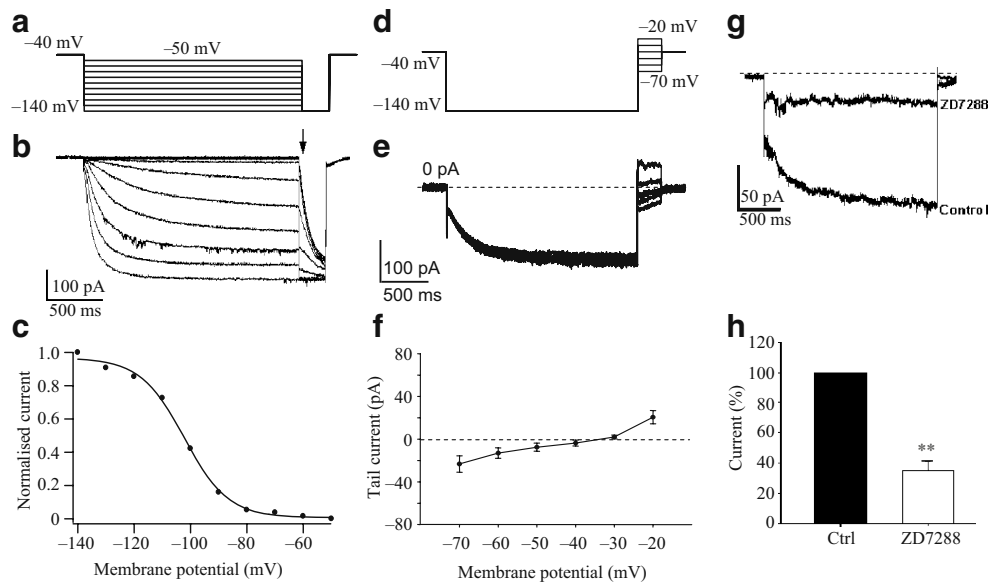
measured at −140 mV immediately after the conditioning voltages (indicated by an arrow in Fig. 2b) were normalised and plotted as a function of the conditioning membrane voltages, then the data were fitted using the Boltzmann equation [28]. As shown (Fig. 2c), under the conditioning voltages, the amplitudes of the peak tail current displayed a typical sigmoidal property [25] and the membrane potential of half-maximal activation ($V_{1/2}$) was found to be −102±1 mV (the slope factor: 7.8±0.1 mV, $n=6$).

To examine the reversal potential, another protocol was used. A pre-hyperpolarising pulse to –140 mV was applied in order to maximally activate HCN channels, and the membrane was then clamped back to test pulses ranging from −70 to −20 mV (Fig. 2d). The tail currents were measured under the test pulses (Fig. 2e) and a current–voltage (I/V) relationship of the fully activated HCN channels was made by plotting the tail currents against the test potentials, and the reversal potential was found to be −33 mV (Fig. 2f). ZD7288, an HCN channel blocker [29] was used to verify the specificity of the HCN channel current in alpha-TC6 cells. As shown, the sustained HCN channel currents obtained from the cells clamped at –140 mV were significantly blocked by 50 μmol/l ZD7288 (Fig. 2g, h; the blockade=65.3±6.8%, $p<0.01$, $n=6$). Therefore, these channels possess typical characteristics of HCN channels as reported [25, 30].

HCN channels modulate glucagon secretion in alpha cell lines To determine the functional role of HCN channels in alpha cells, we measured glucagon release by RIA. Our data showed that high glucose (20 mmol/l) significantly suppressed glucagon release compared with low glucose (2 mmol/l) in alpha-TC6 cells (Fig. 3a, $p<0.05$, $n=4$). Treatment with ZD7288 (100 μmol/l, 30 min) enhanced glucagon secretion by ~65% ($p<0.05$, $n=4$) and ~78% ($p<0.05$, $n=4$) in the presence of either low (2 mmol/l) or high (20 mmol/l) glucose, respectively (Fig. 3a). Similar results were observed with IN-R1-G9 cells (Fig. 3b), another well-established alpha cell line. Conversely, the HCN channel agonist lamotrigine significantly suppressed glucagon secretion from alpha-TC6 cells at 2 mmol/l glucose (Fig. 3c; $n=4$, $p<0.05$), but had no effect on glucagon secretion in the presence of 20 mmol/l glucose. Similar results were also seen with IN-R1-G9 cells (Fig. 3d). These data indicate that modulation of HCN channel activity alters secretory function in alpha cells.

Ca^{2+} and Na^+ channels mediate HCN channel function in alpha-TC6 cells To further establish the mechanism by which alteration of HCN channel activity modulates glucagon secretion, we measured $[Ca^{2+}]_i$ in response to HCN channel blocker ZD7288 in alpha-TC6 cells. As shown, blockade of the HCN channels by ZD7288

Fig. 2 Characterisation of functional HCN channels in alpha-TC6 cells. Cells were clamped from a holding potential of −40 mV to various hyperpolarising conditioning voltages (−140 to −50 mV) followed by a step to −140 mV (**a**). Representative current traces were induced under this voltage protocol (**b**). **c** The tail currents measured at −140 mV immediately after the conditioning voltages (indicated by an arrow in **b**) were normalised and plotted as a function of the conditioning voltages. The $V_{1/2}$ is −102±1 mV (n=6). **d** To determine the I/V relationship of the fully activated HCN channels, the protocol

of a pre-pulse to −140 mV followed by test pulses ranging from −70 mV to −20 mV was used. **e** The representative current traces induced by this protocol. **f** I/V curve was made by plotting the tail currents against the test potentials. The reversal potential is −33 mV (n=6). **g**, **h** Effect of ZD7288 on HCN channels. Representative currents evoked at −140 mV with or without 50 μmol/l ZD7288 treatments (**g**) and the normalised current amplitudes are shown in (**h**) (control [Ctrl] vs ZD7288=100 vs 34.7±6.8, **p<0.01, n=6)
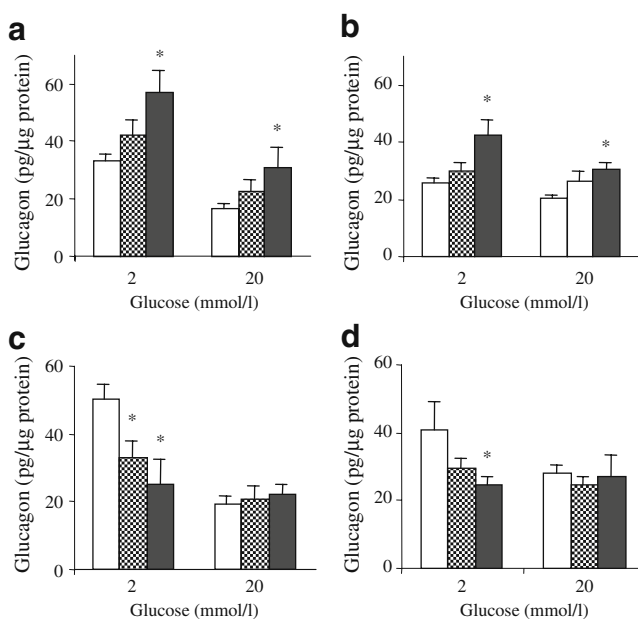

Fig. 3 HCN channels modulate glucagon secretion in alpha cells. Glucagon secretion from alpha-TC6 cells (**a**, **c**) or IN-R1-G9 cells (**b**, **d**) measured by RIA in the presence of different concentrations of ZD7288 (**a**, **b**) or lamotrigine (**c**, **d**) at low (2 mmol/l) or high (20 mmol/l) glucose (white columns, 0 μmol/l; chequered columns, 10 μmol/l; black columns, 100 μmol/l). *p<0.05, n=4 for each group

significantly increased $[Ca^{2+}]_i$ (Fig. 4a), while lamotrigine (10 μmol/l) attenuated the action of ZD7288 on $[Ca^{2+}]_i$ (Fig. 4a). As a positive control, application of depolarising amounts of KCl (30 mmol/l) further enhanced $[Ca^{2+}]_i$. These findings indicate that the effect of HCN channels on glucagon secretion in alpha cells is mediated by modulating $[Ca^{2+}]_i$.

Alpha cells are equipped with Na^+ channels that are critical in the generation of action potential and modulation of glucagon secretion [11, 14, 31]. Therefore, we sought to determine if the Na^+ channel is involved in the process of HCN channel-mediated modulation of glucagon secretion in alpha-TC6 cells. To this end, we determined if blocking the Na^+ channel alters the effect of HCN channels on $[Ca^{2+}]_i$. As shown, blockade of the Na^+ channels by using the specific blocker TTX (0.3 μmol/l) significantly attenuated the elevation of $[Ca^{2+}]_i$ induced by ZD7288 (Fig. 4b,c; ZD7288 vs ZD7288+TTX 100% vs 36%, p<0.001, n=3), suggesting that the HCN channel-induced $[Ca^{2+}]_i$ change is mediated by Na^+ channels in alpha cells.

HCN channels modulate glucagon secretion from rat islets The findings from the clonal alpha cells indicate that modulation of HCN channel activity alters intracellular Ca^{2+}
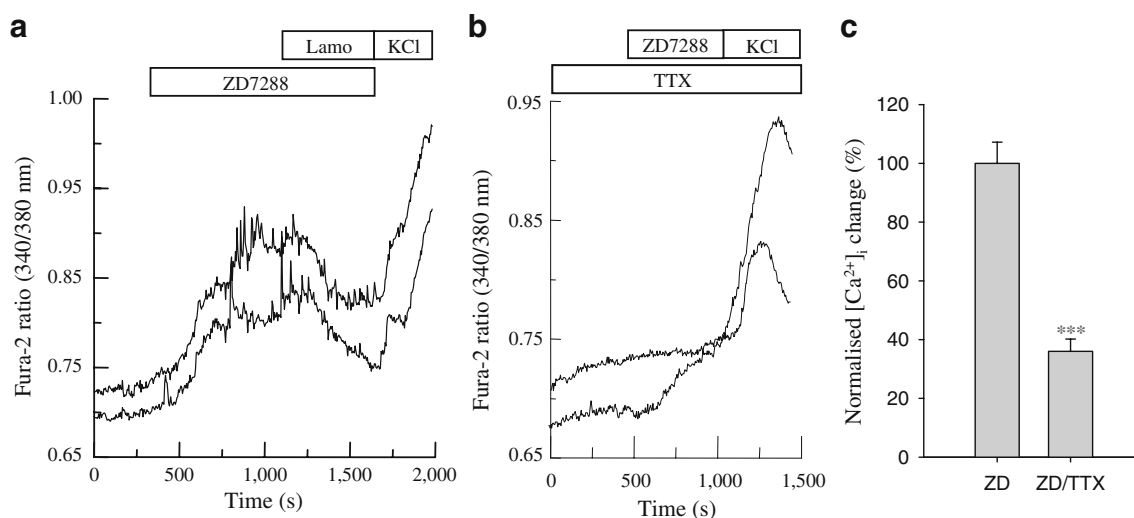
Fig. 4 Ca^{2+} and Na^{+} channels mediate HCN channel function in alpha-TC6 cells. Cells loaded with Fura-2 AM were perfused with buffer containing 1 mmol/l glucose in the absence (a) or presence of 0.3 μmol/l TTX (b) as indicated and were then challenged with 60 μmol/l ZD7288 or plus 10 μmol/l lamotrigine (Lamo) followed by perfusion of 30 mmol/l KCl. Three independent experiments were performed in each case, and 7–15 cells were measured from each experiment. Only those cells that exhibited depolarised responses to KCl were chosen for analysis. For clarity, the representative $[Ca^{2+}]_i$ traces of two individual cells are shown in each case. c The normalised $[Ca^{2+}]_i$ changes induced by ZD7288 (ZD) (a) and ZD7288 plus TTX (b) are shown ($n=3$, ***$p<0.001$)

mobilisation and glucagon secretion. To further determine if these phenomena are of physiological relevance, we performed glucagon secretion RIA using isolated rat islets. Our data showed that treatment of ZD7288 (100 μmol/l, 60 min) significantly enhanced glucagon secretion at 1 mmol/l glucose conditions compared with the control (Fig. 5, 1.44±0.16 vs 0.86±0.19 pg/μg DNA, $p<0.05$), whereas ZD7288 had no significant effect on glucagon secretion under 11 mmol/l glucose (Fig. 5, 0.64±0.06 vs 0.56±0.20 pg/μg DNA, $p>0.05$). We repeated the same experiment with another HCN channel blocker, cilobradine, and found that glucagon secretion was substantially in-

creased at both 1 and 11 mmol/l glucose conditions when treated with 5 μmol/l cilobradine (Fig. 5, $p<0.05$ vs control).

Discussion

We first detected the presence of HCN channels in the alpha-TC6 cell line and rat islet alpha cells. Furthermore, using the patch-clamp technique, the hyperpolarisation-activated inward currents were consistently detected in alpha-TC6 cells. The steady-state voltage activation curve revealed that $V_{1/2}$ was −102±1 mV, which is comparable to those of HCN channels detected in cardiac cells [25] and neuronal cells [30]. The reversal potential of −33 mV obtained from the fully activated inward currents in alpha-TC6 cells suggests that this channel is permeable to both Na^{+} and K^{+} [32], which represents the typical characteristics of HCN channels as reported in other cell types [25, 30, 33]. Additionally, ZD7288, an HCN channel blocker, significantly blocked these currents, indicating that the slow-activating inward currents in alpha cells were indeed from HCN channels.

The physiological functions of HCN channels in alpha cells were then examined in this study. We found that blockade of HCN channels by ZD7288 significantly enhanced glucagon release from both alpha-TC6 cells and IN-R1-G9 cells. We have shown in a previous study that ZD7288 directly exerts an inhibitory effect on exocytosis, in addition to inhibiting HCN channels [26]. We also found that ZD7288 has no effects on
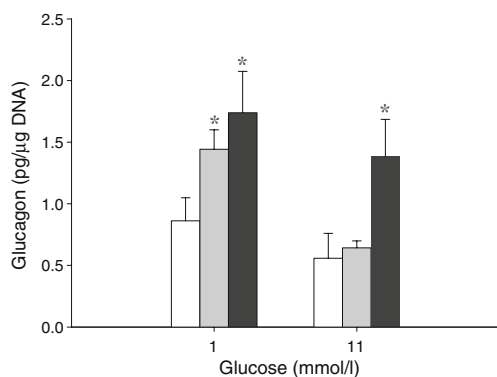
Fig. 5 HCN channels modulate glucagon secretion from rat islets. Glucagon secretion from alpha cells in rat islets was measured at 1 or 11 mmol/l glucose, in the absence (white bars) or presence of ZD7288 (100 μmol/l, grey bars) or cilobradine (5 μmol/l, black bars). *$p<0.05$ vs control, $n=6$
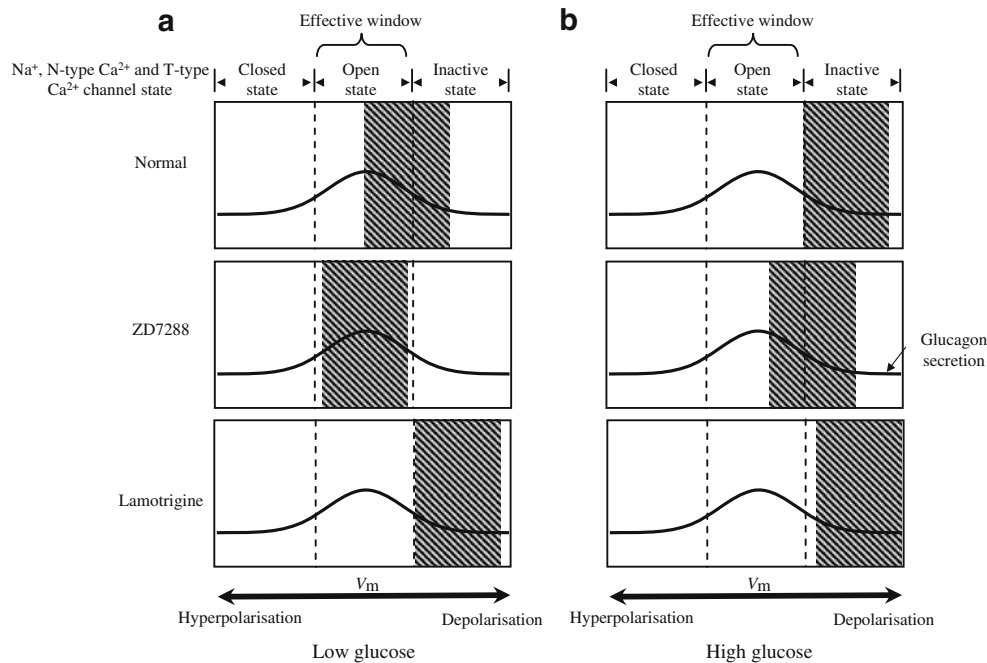
Fig. 6 A model showing HCN channel-mediated regulation of glucagon secretion in alpha cells under normal conditions, or with ZD7288 or lamotrigine treatments. Schematic representation of the effects of HCN channels on alpha cell membrane potential and Na^+ and N- and T-type Ca^{2+} channel activities and glucagon secretion. Alpha cell membrane potential (V_m) change is indicated by the lower arrow bar. Na^+, N-type Ca^{2+} and T-type Ca^{2+} channel activities are shown by their gating states (closed, open and inactive state) at different membrane potential levels. The open state area represents the

alpha cell membrane potential 'window', where glucagon release is triggered through the activation of Na^+ and Ca^{2+} channels. When the alpha cell is too depolarised or too hyperpolarised, the channels then switch to inactive states or closed states, respectively. Under both conditions, no action potential can be generated and glucagon secretion is suppressed. The hatched grey areas represent the 'actual membrane potential' upon involvement of the HCN channels at low (**a**) and high (**b**) glucose concentrations. (see "Discussion" section for details)

voltage-dependent potassium channels, calcium channels and K_{ATP} channels (M. B. Wheeler, unpublished data). Therefore, the stimulatory effect of HCN channel inhibition by ZD7288 on the glucagon secretion might be underestimated because of its direct inhibitory effect on exocytosis. Lamotrigine has been reported as a Na^+ channel inhibitor [34], but this drug activates HCN channels in pyramidal neurons without affecting Na^+ channels [35, 36]. We found that lamotrigine did not significantly influence Na^+ channels in alpha cells at low concentration (10 μmol/l, data not shown), while 10 μmol/l lamotrigine led to suppression of glucagon secretion. These results indicate that alterations in HCN channel activity affect glucagon secretion in alpha cells. To establish the underlying mechanism, we examined the intracellular Ca^{2+} responses in alpha-TC6 cells. As expected, inhibition of the HCN channels by ZD7288 caused a significant increase of $[Ca^{2+}]_i$, while activation of HCN channels by 10 μmol/l lamotrigine attenuated the effects of ZD7288.

Activation of alpha cell voltage-gated Ca^{2+} channels and the resultant glucagon secretion requires the activation of Na^+ channels. Several reports have shown that pharmacological blockade of Na^+ channels inhibits glucagon secretion [11, 14, 18, 31]. Given that we had observed an

augmentation of $[Ca^{2+}]_i$ associated with enhanced glucagon secretion upon inhibition of HCN channels, we hypothesised that Na^+ channels might mediate these effects. To verify this, we have tested the effect of Na^+ channel blocker TTX on ZD7288-induced $[Ca^{2+}]_i$ change. Indeed, we found that TTX could significantly attenuate ZD7288-induced $[Ca^{2+}]_i$ elevation, indicating that the Na^+ channel is downstream of the HCN channel function in alpha cells.

In a more physiological setting, we determined if changes in HCN channel activity alter glucagon secretion in rat alpha cells. Consistently, we found that blockage of HCN by ZD7288 at 1 mmol/l glucose conditions significantly increased glucagon secretion from the rat islets, while no significant effect was observed under 11 mmol/l glucose. The lesser effect of ZD7288 on glucagon secretion at high glucose is probably because of the hyperpolarisation-activated characteristics of HCN channels; they are supposed to be less active when the membranes are depolarised under high glucose concentrations. Interestingly, compared with the effect of ZD7288, we found that cilobradine led to a stronger enhancement of glucagon secretion at both 1 and 11 mmol/l glucose conditions. Given that cilobradine is also a voltage-dependent potassium (K_V) channel inhibitor [26] and that,

blockade of K_V channel causes enhancement of cellular secretion [12], it is possible that the profound effect of cilobradine on glucagon secretion is probably via both HCN channel and K_V channel pathways. However, because of the complicated paracrine interactions among islet alpha, beta and delta cells, it is difficult to make a precise interpretation of the results obtained from islet cells. Nevertheless, the consistent findings that inactivating HCN channels increases glucagon secretion from clonal glucagon-secreting cells and isolated rat islets suggest an important role for HCN channels in modulating glucagon secretion from alpha cells.

It is interesting that in pancreatic beta cells, HCN channels do not seem to play a crucial role in modulating insulin secretion [26]. The difference might be attributed to the unique biophysical properties of alpha cells. Numerous studies have demonstrated that: (1) alpha cell K_{ATP} channels are more sensitive to ATP inhibition than those in beta cells; thus, the K_{ATP} channels in alpha cells possess intermediate activity under low glucose concentrations [3, 11, 31, 37]; (2) unlike the beta cell Na^+ channels, which are activated at a more negative membrane potential, the alpha cell Na^+ channels are active within a physiological range of membrane potentials [14, 17, 31]; and (3) in addition to the L-type Ca^{2+} channels that are the most important in modulating $[Ca^{2+}]_i$ in beta cells, the activities of T-type and N-type Ca^{2+} channels in alpha cells are critical in the generation of action potentials and control of glucagon secretion [11, 14, 15, 38]. Based on these findings, it has been proposed that glucagon secretion only occurs within a narrow window of intermediate alpha cell K_{ATP} channel activity (e.g. under low glucose conditions), where the membrane potential elicits regenerative electrical activities through the activation of Na^+ and T- and N-type Ca^{2+} channels [10, 11, 14].

The present study suggests a potential role for HCN channels in regulation of glucagon secretion via modulating the regenerative electrical activities in alpha cells (Fig. 6). It is likely that under low glucose conditions the inward HCN currents cause a slight alpha cell depolarisation that leads to inactivation of a small fraction of the Na^+ and T- and N-type Ca^{2+} channels, partly suppressing glucagon secretion (grey hatched in Fig. 6a, upper panel). While with treatment of ZD7288, the blockade of inward HCN currents causes a slight membrane hyperpolarisation, this would release more Na^+ and Ca^{2+} channels from their inactive state, making them available to be activated, and as a consequence, increasing alpha cell electrical activity and glucagon secretion (grey hatched in Fig. 6a, middle panel). Conversely, the enhancement of inward HCN currents induced by lamotrigine further depolarises the membrane to a level that is out of the effective window, resulting in the inactivation of Na^+ and T- and N-type Ca^{2+} channels and the suppression of glucagon secretion (grey hatched in

Fig. 6a, lower panel). Given the hyperpolarisation-activated characteristics of HCN channels, they would be anticipated to be less active under high glucose concentrations (i.e. membrane depolarisation conditions) (grey hatched in Fig. 6b). Nevertheless, under high glucose concentrations, inactivation of HCN channels still resulted in an induction of glucagon secretion.

The physiological relevance of HCN channels in alpha cells is at present not clear. It is known that paracrine factors including insulin, zinc and GABA play important roles in modulating glucose-regulated glucagon secretion [39]. A recent study suggested that glutamate released from alpha cells exerts autocrine effects to produce adequate glucagon release under hypoglycaemic conditions [40]. Given that, physiologically, a negative feedback simultaneously occurs during a secretory process, it is possible that the HCN channel in alpha cells may function as a negative feedback modulator to prevent an 'overshooting' of glucagon secretion under glucagon-stimulatory conditions, hence to maintain the hormone at appropriate levels.

It now remains to be seen how HCN channel activation is affected under physiological conditions. Our experiments have shown that the increase of intracellular cAMP only caused a 3 mV right shift of the voltage activation curve for HCN channels in alpha-TC6 cells (data not shown), and it appears that cAMP has less effect in modulating the activity of HCN channels in alpha-TC6 cells. Considering the recent report that phosphatidylinositol 4,5-bisphosphate activates HCN channels in *Xenopus* oocytes via a cAMP-independent pathway [41], and that elevation of cAMP initiates multiple pathways with divergent components, further investigations to determine the precise molecular mechanism that underlies HCN channel regulation in alpha cells are required and will provide new information and potential therapeutic targets to optimise the treatment of diabetes.

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