**Blunted excitability of aortic baroreceptor neurons in diabetic rats: involvement of hyperpolarization-activated channel**

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**Aims** Although dysfunction of arterial baroreflex occurs in human and animal models of type-1 diabetes (T1D), the mechanisms involved in the impairment of the baroreflex still remain unclear. The nodose ganglion (NG) contains the cell bodies of the aortic baroreceptor (AB) neurons. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in AB neurons and play an important role in regulating the cell excitability. We investigated whether the excitability of AB neurons is depressed in streptozotocin (STZ)-induced T1D rats and whether HCN channels are involved in this depression.

**Methods and results** Using the whole-cell patch clamp technique, we found that AB neuron excitability (action potential frequency at 50 pA current stimulation) in the T1D rats was lower than that in the sham rats (0.4 ± 0.5 vs. 4.8 ± 0.6 spikes/s, P < 0.05; AB neurons were identified by DiI staining). In addition, HCN current density in AB neurons from the T1D rats was bigger than that from the sham rats (60.2 ± 6.1 vs. 30.7 ± 4.9 pA/pF at test pulse +140 from holding potential −40 mV, P < 0.05). Furthermore, HCN channel blockers (5 mM cesium chloride and 100 μM ZD7288) significantly reduced HCN currents and increased action potential frequency of the AB neurons in sham and T1D rats. Immunofluorescent and western blot analyses demonstrated that the expression of HCN1 and HCN2 channel protein in the NG from the T1D rats was higher than that from the sham rats.

**Conclusion** These results indicate that the HCN channels influence the excitability of AB neurons, and more importantly, contribute to the decreased excitability of AB neurons in T1D rats.

**KEYWORDS** Electrophysiology; Ion channels; Baroreflex; Autonomic nervous system; Diabetes

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**1. Introduction**

Diabetes is a major world health problem. Baroreflex dysfunction is a frequent complication of diabetes, which may contribute to the increase of the mortality rate in diabetic patients. The arterial baroreflex normally acts to prevent wide oscillations in blood pressure (BP), acting on both the sympathetic and parasympathetic limbs of the autonomic nervous system. A population of neurons whose cell bodies are contained within the nodose and petrosal ganglia serves as a primary afferent limb of the baroreceptor reflex. These baroreceptor neurons sense BP by increasing their discharge (excitation) when BP rises and then decrease heart rate (HR) via the baroreflex arc. Baroreflex sensitivity is reduced in type-1 diabetic (T1D) patients even without overt autonomic neuropathy. Control of the heart is diminished in T1D patients as evidenced by the reduced beat-to-beat variation in HR.

Function is decreased in short- and long-term streptozotocin (STZ)-induced T1D in rats. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels were discovered in sino-atrial cells and in hippocampal pyramidal cells and are usually associated with cardiac pacemaker activity or neuron oscillatory behaviour. However, HCN channels are also expressed in the soma of visceral-sensory and somato-sensory neurons that generally exhibit neither pacemaker activity nor oscillatory potentials. Generally somato-sensory and visceral-sensory cell bodies are quiescent except in response to a depolarizing stimulus generated by their peripheral sensory receptors. HCN channels play a different role in these non-oscillatory neurons. One recent study has demonstrated that inhibition of HCN channels in nodose ganglion (NG) afferent neurons decreases the threshold to evoke an action potential in response to a depolarizing stimulus, rendering the neurons more excitable.

The HCN channel has been divided into four isoforms (HCN1–4) with respect to their cAMP sensitivity and the time and voltage dependence of activation. mRNA for...
HCN1, HCN2, HCN3, and HCN4 are expressed in rat NG neurons.\(^2\) Furthermore, HCN1 is present in the neurons with myelinated A-fibres but not the neurons with unmyelinated C-fibres, whereas HCN2 and HCN4 are present in both A-fibre and C-fibre nodose neurons.\(^2\) These different patterns of localization of the isoforms suggest that different HCN isoforms may play distinct roles (such as, inward current with rapid activation in A-fibre and with slow activation in C-fibre)\(^2,26\).

The aortic depressor nerve (ADN) is composed of both afferent A-fibres (about 25%) and C-fibres (about 75%).\(^2\) There are very different dynamic sensory discharge characteristics between A-fibre and C-fibre baroreceptor afferents. C-fibre afferents are activated mainly at very high pressure and have lower firing frequencies, irregular discharge patterns,\(^2\) and appear to be the primary regulators of tonic baseline levels of BR.\(^2\) A-fibre afferents have lower pressure thresholds with very stable, proportional firing patterns,\(^2\) which are thought to regulate the baroreflex sensitivity but not baseline levels of BR.\(^2\) We hypothesize that HCN channel over-expression and hyperactivation blunt the excitability of A-fibre aortic baroreceptor (AB) neurons in T1D rats. To test this hypothesis, we determined whether the excitability of A-fibre AB neurons is depressed in the NG from T1D rats, and whether HCN channels are involved in this depression.

2. Methods

2.1 Induction of diabetes

Male Sprague-Dawley rats (200-280 g) were housed two per cage under controlled temperature and humidity and a 12:12 h dark-light cycle, and were provided water and rat chow ad libitum. Experiments were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee and were carried out in accordance with the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the American Physiological Society’s Guides for the Care and Use of Laboratory Animals.

Rats were randomly assigned to sham (n = 25) and T1D (n = 25). Diabetes was induced by a single intraperitoneal injection of STZ (65 mg/kg, Sigma) in a 2% solution of 0.1 M cold citrate buffer. Sham rats received a similar injection of vehicle. Diabetes was identified by polydipsia, polyuria, and blood glucose > 250 mg/dL (Accu-check Aviva, Boehringer Mannheim). Rats receiving STZ but with blood glucose < 250 mg/dL (n = 2) were excluded from study. Blood glucose and body weight in all rats were measured weekly. All experiments were taken at 6-8 weeks after single-dose injection of STZ or vehicle.

2.2 Labelling of aortic baroreceptor neurons

Since there are only baroreceptor afferent fibres and no chemoreceptor afferent fibres in rat ADN innervating the aortic arch,\(^2\) the AB neurons in the NG were selectively retrograde-labelled by a transported fluorescent dye, Dil (red colour, Molecular Probes, Eugene, OR). Two weeks before the final experiments, the rats were anesthetized with isoflurane at 2% and mechanically ventilated. A thoracotomy was made at the third intercostal space. With gentle dissection, the pulmonary artery was retracted from the aorta, exposing the lesser curvature of the aortic arch. Dil (2 μL) was injected into the adventitia of the aortic arch with a fine-tipped glass pipette. After application of the dye, the surgical incision was closed and negative intrathoracic pressure was re-established. The rat was allowed to recover for at least 1 week to allow the dye to diffuse to the AB neurons in the NG. All procedures were performed under sterile conditions.\(^2\)

2.3 Isolation of nodose sensory neurons

The region of the NG was excised 6-8 weeks after single-dose STZ (n = 13) or vehicle (n = 15) injection. Both NGs in each rat were removed and placed in ice-cold Ringer’s solution (mM): NaCl, 137; NaHCO\(_3\), 25; KCl, 3; NaH\(_2\)PO\(_4\), 1.25; CaCl\(_2\), 1.2; MgSO\(_4\), 1.2; glucose, 10. The NGs were dissected free and incubated for 30 min at 37 °C in an enzymatic Ringer’s solution containing 0.1% collagenase/0.1% trypsin. The tissues were mechanically triturated and then transferred to a Ringer’s solution containing 0.2% collagenase and 0.5% bovine serum albumin (BSA) incubated for 30 min at 37 °C. After digestion, dispersed NG cells were washed in Dulbecco’s modified Eagle’s medium (DMEM) containing 6% BSA. Cell suspension was plated onto a tissue culture dish and kept at 37 °C in an incubator with a humidified atmosphere of 95% air-5% CO\(_2\) for 4-24 h before the neurons were used in the patch-clamp experiments.

2.4 Recording of whole-cell hyperpolarization-activated cyclic nucleotide-gated currents and action potentials

Only Dil-labelled NG neurons (AB neurons) were selected for electrophysiological study. HCN currents and cell action potentials were recorded by the whole cell patch-clamp technique using Axopatch 200B patch-clamp amplifier (Axon Instruments, Inc.). A switch on the amplifier allows the same cell to be recorded in either the voltage-clamp for HCN currents or current-clamp mode for cell membrane potential. Resistance of the patch pipette was 1-3 MΩ when filled with (in mM) 145 K-aspartate, 5 NaCl, 1.95 CaCl\(_2\), 2.2 EGTA, 2 MgCl\(_2\), 5 HEPES, and 10 glucose, pH 7.3. The extracellular solution consisted of (in mM): 137 NaCl, 5.4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 HEPES, 10 glucose with pH 7.4. Current traces were sampled at 10 kHz and filtered at 5 kHz. In the voltage-clamp experiments, holding potential was −40 mV. Current–voltage (I-V) relations were elicited by 200 ms step decrements to potentials between −40 and −140 mV for 1 s followed by a step to −80 mV for 0.5 s before returning to holding potential.\(^2\) Peak currents were measured for each test potential and current density was calculated by dividing peak current by cell membrane capacitance (C\(_m\)). The data points of current density were plotted against the corresponding test potential. Time constants (τ1 and τ2) were calculated by a biexponential function: \[ I(t) = A_0 + A_1 \exp(-t/τ_1) + A_2 \exp(-t/τ_2), \]

where \( A_0 \) is the offset constant, \( A_1 \) and \( A_2 \) are the amplitudes, \( τ_1 \) and \( τ_2 \) are the respective time constants. The membrane potential was returned to −80 mV in voltage protocol to measure the tail currents.\(^2\) An activation curve was constructed from the current-voltage (I-V) relations and fitted to the Boltzmann function:

\[ \frac{1}{1 + \exp(V-V_{1/2})/K}], \]

where \( V_{1/2} \) is the normalized tail current at a given test potential \( V \), equaling \( (I-I_{-\infty})/(I_{-∞}-I_{-∞}) \), \( I_{-∞} \) is the maximal tail current; \( V_{1/2} \) is the voltage for half-activation, and \( K \) is the slope factor. In the current-clamp experiments, the action potential was recorded at a current injection of 10-100 pA. The following procedures were used to identify the type of afferent neurons (A-fibre vs. C-fibre). At the completion of data collection, cells were held at −80 mV and stepped to depolarizing potentials in 10 mV increments to induce the fast transient sodium currents (I\(_{Na}\)). The cells were then perfused 1 μM TTX (in the extracellular bath and the test protocol was repeated. The inhibited I\(_{Na}\) was defined as TTX sensitive (TTX-S) and the remaining I\(_{Na}\) as TTX resistant (TTX-R). C-fibre neurons express both TTX-S and TTX-R but A-fibre neurons express only TTX-S.\(^2\) In order to reveal the possible mechanisms that contribute to the reduced baroreflex sensitivity in diabetes, the cell excitability and HCN currents of A-fibre AB neurons were investigated in this study. P-clamp 9.2 programs (Axon Instruments) were used for data acquisition and analysis. All experiments were done at room temperature. In order to measure the effect of each treatment, 8 A-fibre AB neuron cells from eight rats were used in each group.
2.5 Protocol for action potential and hyperpolarization-activated cyclic nucleotide-gated current recording

Experiment 1: Action potential and HCN currents were recorded before and after perfusing cesium chloride (CsCl) or ZD7288 in A-fibre AB neurons from sham (n = 8) and diabetic (n = 8) rats. Although CsCl has been reported to inhibit other potassium currents such as the inward rectifier potassium currents, the delayed rectifier potassium currents, there is no functional expression of the inward rectifier potassium channel in rat NGs. ZD7288 has no effect on the total outward potassium currents. Therefore, CsCl can be a tool to specifically inhibit HCN channels in rat NGs. In addition, another specific HCN channel blocker, ZD7288, was also used in this study.

Experiment 2: HCN currents were recorded before and after perfusing 8-Br-cAMP in A-fibre AB neurons from sham (n = 8) and diabetic (n = 8) rats to investigate sensitivity of HCN channels to cAMP. Concentration of CsCl, ZD7288, and 8-Br-cAMP was used based on previous studies and our preliminary study.

2.6 Western blot analysis for HCN1 and HCN2 channel protein

NGs from sham (n = 7) and diabetic (n = 7) rats were rapidly removed and immediately frozen in liquid nitrogen and stored at −80°C until analysed. The homogenates were prepared from NG samples. The protein was extracted with the lysing buffer [10 mM Tris, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 7.4] plus protease inhibitor cocktail (Sigma, 100 µL/mL). After centrifugation at 12,000g for 20 min at 4°C, the protein concentration in the supernatant was determined using a bicinchoninic acid protein assay kit (Pierce; Rockford, IL). The protein sample was mixed with the same volume of the loading buffer and heated at 100°C for 20 min. Equal amounts of the protein samples were loaded and then separated on a 10% SDS-polyacrylamide gel. The proteins of these samples were electrophoretically transferred at 300 mA for 1.5 h onto PVDF membrane. The membrane was probed with rabbit anti-HCN1 and -HCN2 channel antibodies (Alomone labs) and a peroxidase-conjugated goat anti-rabbit IgG (Pierce Chemical, Rockford, IL). The signal was detected using enhanced chemiluminescent substrate (Pierce Chemical, Rockford, IL) and the bands were analysed using UVP bioimaging system. The target protein was controlled by probing western blot with mouse anti-GAPDH antibody (Santa Cruz, CA) and normalizing target protein intensity to that of GAPDH.

2.7 Immunofluorescence for HCN1, HCN2, and RT 97 detection

Immunofluorescent staining was taken in sham (n = 3) and diabetic (n = 3) rats. Each rat was perfused transcardially with 100 mL heparinized saline followed by 500 mL of freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PBS). Both NGs in each rat were rapidly removed and postfixed in 4% paraformaldehyde in 0.1 M PBS for 12 h at 4°C, followed by soaking the NG in 30% sucrose for 12 h at 4°C for cryostat protection. The NG was cut into 10 µM-thick sections and then mounted on pre-coated glass slides. After 30 min in PBS, the NG sections were incubated with 10% goat serum for 1 h followed by incubation with rabbit anti-HCN1 or -HCN2 (Alomone Laboratories, Israel) and mouse anti-RT 97 antibodies (a A-fibre neuron marker, Abcam, Cambridge, MA) overnight at 4°C. Then the sections were washed with PBS and incubated with fluorescence-conjugated secondary antibody for 60 min at room temperature. After three washes with PBS and air-drying, the sections were mounted on pre-cleaned microscope slides. Slides were observed under a Leica fluorescent microscope with corresponding filters. Pictures were captured by a digital camera system. No staining was seen when PBS was used instead of the primary antibody in the above procedure.

2.8 Drugs

ZD7288 was purchased from Tocris Bioscience, MO, USA. Other chemicals used in this study were obtained from Sigma-Aldrich Chemical Co., MO, USA.

2.9 Data analysis

All data are presented as mean ± SEM. SigmaStat 3.5 was used for data analysis. Statistical significance was determined by Student’s unpaired t-test for body weight, blood glucose, and protein expression of HCN channel isoforms. A two-way ANOVA, with a post hoc test, was used in comparisons of HCN currents and action potential of NG A-fibre neurons. All data were confirmed by the Kolmogorov-Smirnov test to fit reasonably within normal distribution and equal variance was confirmed by the Levene test. Statistical significance was accepted when P < 0.05. A power analysis was conducted to assess whether the sample size was sufficient to ensure P < 0.05.

3. Results

3.1 Induction of diabetes

Diabetes was induced by single-dose injection of STZ (65 mg/kg). After 6-8 weeks of STZ injection, the mean blood glucose was significantly higher (424 ± 28 mg/dL, n = 23), compared with that in sham rats (vehicle injection, 98 ± 16 mg/dL, P < 0.05, n = 25). The body weight in T1D rats was lower than that in sham rats (231 ± 19 vs. 360 ± 17 g, P < 0.05).

3.2 Membrane excitability in DiI-labelled A-fibre aortic baroreceptor neurons from sham and type-1 diabetic rats

As shown in Figure 1A, AB neurons were labelled by Dil and identified by the microscope with fluorescent filter. A-fibre neurons were identified according to the presence of only TTX-S-sensitive Na+ currents, as well as action potential frequency of the AB neurons in sham and T1D rats towards the same level (Figure 1). The number of action potential induced by 50 pA current injection in A-fibre AB neurons from T1D rats was lower than that in sham rats (0.4 ± 0.5 vs. 4.8 ± 0.6 spikes/s, P < 0.05, Figure 1). CsCl and ZD7288 significantly increased action potential frequency of the AB neurons in sham and T1D rats (Figure 1).

In addition, the resting membrane potential (RMP) of the A-fibre AB neurons in T1D rats (−51.4 ± 1.2 mV) was depolarized (P < 0.05), compared with that in sham rats (−61.9 ± 1.1 mV). After treatment with CsCl or ZD7288, the RMP was hyperpolarized (P < 0.05 vs. pre-treatment) in sham (−75.6 ± 1.3 and −74.4 ± 1.0 mV) and diabetic (−72.3 ± 1.2 and −73.5 ± 1.4 mV) rats.
3.3 Hyperpolarization-activated cyclic nucleotide-gated currents in DiI-labelled A-fibre aortic baroreceptor neurons from sham and type-1 diabetic rats

There was no significant difference in the whole-cell membrane capacitance between sham and T1D rats (51.4 ± 1.0 vs. 53.3 ± 1.1 pF, n = 40 cells from 15 sham or 13 diabetic rats, P > 0.05).

Figure 2 illustrates typical HCN current recordings and I–V curves obtained from A-fibre AB neuron cells from sham and T1D rats. In sham rats, when the neuron cell was hyperpolarized from −240 mV hold potential with 10 mV step decrements, the HCN current (a slow non-inactivating inward current) increased. At −140 mV, the fast time constant (τ₁) was 63 ± 6 ms and the slow time constant (τ₂) was 452 ± 29 ms. In T1D rats, HCN current density was significantly increased, compared with that from the sham rats (P < 0.05, Figure 2). The neuron cells had 173 ± 23 ms for τ₁ and 302 ± 26 ms for τ₂ at −140 mV (P < 0.05 vs. sham rats). Furthermore, CsCl and ZD7288 markedly reduced HCN currents in sham and T1D rats (Figure 2).

3.4 Sensitivity of hyperpolarization-activated cyclic nucleotide-gated channels to cAMP in DiI-labelled A-fibre aortic baroreceptor neurons from sham and type-1 diabetic rats

The effect of cAMP on HCN channels was investigated by application of 8-Br-cAMP (a membrane permeable form of cAMP) to the recording bath. As shown in Figure 3, 100 μM 8-Br-cAMP significantly increased HCN currents in T1D rats but not in sham rats. Using HCN tail current to construct the activation curve, we found that V₁/₂ for the activation curve was 85.2 ± 1.5 and 84.6 ± 1.6 mV, respectively, before and after treatment of 8-Br-cAMP in A-fibre AB neurons from sham rats (P > 0.05). In T1D rats, however, 8-Br-cAMP significantly shifted the activation curve to the right (Figure 3). V₁/₂ for activation curve was changed from 84.2 ± 1.6 in control to 72.7 ± 1.3 mV in the presence of the 8-Br-cAMP (P < 0.05).

3.5 Protein expression of hyperpolarization-activated cyclic nucleotide-gated channel isoforms in DiI-labelled A-fibre aortic baroreceptor neurons from sham and type-1 diabetic rats

Using a double-labelling technique, we found that HCN1 channel protein was mainly localized to the A-fibre...
neurons (Figure 4), which was validated by an immunofluorescent staining marker (RT97) for the A-fibre neurons. Compared with sham rats, immunofluorescent staining for HCN1 channel protein was enhanced in the A-fibre neurons from T1D rats (Figure 4). Similarly, western blot data also showed that HCN1 channel protein was increased in NG from T1D rats vs. sham rats (Figure 4).

There was no significant immunofluorescent staining for HCN2 channel protein in A-fibre neurons from sham rats (Figure 5). However, HCN2 channel immunofluorescent staining was elevated in the A-fibre neurons from T1D rats (Figure 5). Simultaneously, from western blot experiment, the expression of HCN2 channel protein was higher in NG from T1D rats than that in sham rats (Figure 5).

4. Discussion

The major findings from the present study are: (1) A-fibre AB neuron excitability was suppressed and HCN current density was elevated in the NG from T1D rats, compared with that in sham rats; (2) HCN channel blockers (CsCl and ZD7288) decreased the HCN current density and enhanced the cell-membrane excitability via hyperpolarizing RMP and decreasing current threshold in A-fibre AB neurons from sham and T1D rats; (3) T1D significantly increased the sensitivity of
HCN channels to cAMP in A-fibre AB neurons; (4) the protein expression of HCN1 and HCN2 channel isoforms was higher in the NG from T1D rats than that from sham rats. These results suggest that the HCN channels influence the excitability of AB neurons, and more importantly, contribute to the blunted excitability of AB neurons in T1D rats. Overexpression of HCN2 channel protein may be involved in the enhanced sensitivity of HCN channels to cAMP in A-fibre AB neurons from T1D rats.

Previous studies have shown that the functional properties of the arterial baroreflex are blunted in T1D patients and rats. Diabetic patients with abnormal baroreflex have a higher incidence of mortality than those with normal baroreflex function. However, the mechanisms responsible for the impairment of the baroreflex are still poorly understood. The nodose and petrosal ganglia contain the cell bodies of AB and carotid baroreceptor neurons, which serve as the primaryafferent limb of the baroreceptor reflex. The ADN is the AB afferent nerve, which is composed of A-fibre and C-fibre. Recent study has shown that in the intact natural baroreflex, A-fibre baroreflex gain is adequate to produce a prompt and sufficient depressor response and the pressure does not reach the C-fibre activating level because A-fibre ABs have lower threshold and C-fibre ABs are activated at a very high pressure; only when A-fibre gain is insufficient does the BP rise to where the C-fibres are activated to play a role on regulating BP. The present study documents for the first time that T1D significantly suppressed the cell-membrane excitability in the A-fibre AB neurons. Thus, the depressed cell-membrane excitability in the A-fibre AB neurons is likely to be involved in the blunted baroreflex sensitivity observed in diabetic patients and rats. Although our present study does not touch upon the changes of the C-fibre AB neurons in T1D rats, we recognize that our present results cannot rule out the involvement of C-fibre AB neurons in the blunted baroreflex sensitivity of the diabetic patients and animals. Further studies are needed to define the role of C-fibre AB neurons on the modulation of the baroreflex in the diabetics.

HCN channels have been found in cardiac muscle cells, which usually are responsible for the cardiac pacemaker activity, and in central neurons to associate with the oscillatory behaviour. Four members of HCN channels (HCN1, HCN2, HCN3, and HCN4) are also expressed in the rat NG. However, HCN channels may have a different role in the NG neurons, the non-oscillatory cells because the NG neurons are quiescent except in response to a depolarizing stimulus generated by their peripheral sensory receptors. In the NG neurons, the RMP is about −50 to −65 mV, in which voltage-dependent sodium, calcium, and potassium channels are almost inactivated. The inactivation of these voltage-dependent channels can be recovered to the activation state during the hyperpolarization of the RMP, which means the number of available voltage-dependent channels for activation is increased if the NG neurons receive the depolarizing stimulus. Inhibition of HCN channels has been shown to hyperpolarize the NG neurons (increasing the RMP) and to reduce action-potential threshold in response to a depolarizing current stimulation, thus rendering the NG neurons more excitable. Our present study confirms that the HCN current density in A-fibre AB neurons from the T1D rats is larger than that from the sham rats. In addition, the RMP was depolarized and the current-threshold-induced action potentials were elevated in the A-fibre AB neurons from T1D rats, compared with that in sham rats. Furthermore, HCN channel blockers (CsCl and ZD7288) lowered the HCN current density, hyperpolarized the RMP, and raised the cell-membrane excitability in A-fibre AB neurons from sham and T1D rats. These results clearly indicate that the HCN channels are involved in the regulation of A-fibre AB neuron excitability, and the enhancement of HCN currents can contribute to the blunted A-fibre AB neuron excitability in T1D rats.

It was evident from our experiments that the increase of action potential frequency of the AB neurons induced by CsCl and ZD7288 is less in the diabetic rats than in the sham rats (Figure 1). It is possible that the changes of other ion channels could be involved in the depressed cell excitability in T1D neurons. Therefore, further study is needed to explore the other ion-channel characteristics in the diabetic AB neurons.

One of the HCN channel properties is that the activity of the HCN channels is directly enhanced by cAMP-direct-binding to the HCN channels. Some studies have found that the sensitivity of HCN channels to cAMP is higher in C-fibre neurons than that in A-fibre neurons because the HCN1 channel isoform is only located in A-fibre neurons and is relatively insensitive to cAMP, whereas HCN2 is highly sensitive to cAMP and the co-expression of HCN1 and HCN2 can enhance the sensitivity of HCN channels to cAMP. Our present study shows that HCN currents have the activation kinetics of HCN1 (rapid activation) and are not affected by 8-Br-cAMP in A-fibre AB neurons from sham rats (Figures 2 and 3). Immunofluorescent data also show that HCN1 but not HCN2 is mainly located in the A-fibre neurons from sham rats (Figures 4 and 5). These results further indicate that A-fibre AB neurons are insensitive to cAMP only due to HCN1 expression. However, T1D induced the overexpression of HCN1 and HCN2, and enhanced sensitivity of HCN channels to cAMP in A-fibre AB neurons (Figures 3–5). From these data, we propose that HCN currents are markedly enhanced via increasing the numbers of HCN channels and sensitivity of HCN channels to cAMP in A-fibre AB neurons from T1D rats. The enhanced HCN currents then contribute to the blunted cell-membrane excitability in the diabetic A-fibre AB neurons.

Although expression of HCN1 and HCN2 channel proteins is increased in A-fibre NG neurons from diabetic rats (Figures 4 and 5), we cannot clearly confirm overexpression of HCN channels in A-fibre AB neurons from diabetic rats due to the limitation of the method (Dil labelling was lost from cells during the immunofluorescent staining procedure and could not be used as a marker of AB neurons with immunofluorescent staining). However, it is reasonable to assume that HCN1 and HCN2 channel proteins are overexpressed in A-fibre AB neurons from diabetic rats because A-fibre AB neurons are a component of A-fibre neurons in NGs and electrophysiological data indicate that the HCN channel density and the sensitivity of HCN channels to cAMP are enhanced in A-fibre AB neurons from diabetic rats.

In conclusion, overexpression of HCN1 and HCN2 can enhance a hyperpolarizing current through an increase in HCN channel number and sensitivity of HCN channels to cAMP in the A-fibre AB neurons from T1D rats. This enhanced HCN channel function mediates the depressed A-fibre AB neuron excitability and may contribute to the blunted baroreflex sensitivity in diabetic state.
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