Is ZD7288 a selective blocker of hyperpolarization-activated cyclic nucleotide-gated channel currents?

Xing Wu,1 Liping Liao,1 Xiangming Liu,2 Fang Luo,2 Tianming Yang4 and Chenhong Li3,*

1Key Laboratory of Molecular Biophysics of Ministry of Education; College of Life Science and Technology; Wuhan, China; 2Department of Anesthesiology; Tongji Hospital; Huazhong University of Science and Technology; Wuhan, China; 3The Laboratory of Membrane Ion Channels and Medicine; College of Biomedical Engineering; Wuhan, China; 4College of Pharmacy; South-Central University for Nationalities; Wuhan, China

Keywords: ZD7288, HCN channel currents, Na+ currents, neuropathic pain, DRG

ZD7288 has been widely used as a tool in the study of hyperpolarization-activated cyclic nucleotide-gated channels (HCN channels), and to test the relationships between HCN channels and heart and brain function. ZD7288 is widely considered a blocker of HCN channel currents. Here we show that ZD7288 inhibits not only HCN channel currents, but also Na+ currents in DRG neurons and ZD7288 was confirmed to inhibit Na+ current in HEK293 cells transfected with Na1.4 plasmids. Thus our findings challenge the view that ZD7288 is a selective blocker of HCN channels. Conclusions about the role of NCN channels in neuronal function should be re-evaluated if based exclusively on the effect of ZD7288.

Introduction

Since Brown discovered that the mechanism by which adrenaline accelerates heart rate involves hyperpolarization-activated cation currents (HCN) in 1979,1 researchers started to look for the blockers of HCN. After more than 10 y, ZD7288 [4-(N-Ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride ICI-D7288 N-Ethyl-1,6-dihydro-1,2-dimethyl-6-(methylimino)-N-phenyl-4-pyrimidinamine hydrochloride] was found to be a highly selective blocker of HCN.2 ZD7288 reduces heart rate without impairing cardiac function, and it also mediates analgesia in neuropathic pain,3 consistent with an action on HCN channels. Thus, ZD7288 has been widely used as a selective HCN inhibitor in physiological studies. Here, while investigating the effect of HCN currents on neuropathic pain, we discovered that ZD 7288 also blocks Na+ currents. This finding challenges the view that ZD7288 mediates analgesia via block of HCN channels.

Results

Identification of Na+ currents in DRG neurons. Na+ currents recorded in DRG neurons were identified by lidocaine, a special blocker of Na+ currents. As shown in Figure 1A, lidocaine almost completely inhibited sodium currents. The current inhibited could be recovered partially by washout. Protocols used to isolate sodium currents were identical to those described previously.5

ZD7288 inhibits Na+ currents in DRG neurons. Na+ currents were recorded in DRG neurons as described above, and the effects of ZD7288 were investigated. We found that ZD7288 inhibited sodium currents and its effects could be recovered upon washout of ZD7288. Figure 2A1-A4 shows the inhibitory effect of ZD7288 on Na+ currents at different drug concentrations. We also measured the time course (Fig. 2B1-B4) of development of ZD7288 block at various drug concentrations at a test potential of -70 mV. Based on the data of Figure 2, we constructed a dose response curve (Fig. 3) which, when fitted with the Hill equation, yielded an IC50 of 1.17 μM.

ZD7288 does inhibit HCN channel currents. To ensure that ZD7288 was indeed also a blocker of HCN channel currents, we determined the effect of this drug on HCN currents in DRG neurons. HCN channel whole cell current in DRG neurons was induced by giving a membrane potential step from a holding potential of -10 mV, showing that the rate of development of block increased with drug concentration. Based on the data of Figure 2, we constructed a dose response curve (Fig. 3) which, when fitted with the Hill equation, yielded an IC50 of 1.17 μM, indicating that HCN channels are blocked less effectively than sodium channels.

Inhibition of transiently expressed Na1.4 channels by ZD7288. To confirm the inhibitory effects of ZD7288 on sodium channels, we transfected HEK293 with Na1.4 plasmids along with a GFP marker. Na1.4 channel currents were recorded as Figure 5 in absence, presence and following washout of 30 μM ZD7288. As evident from Figure 5, ZD7288 also inhibited transiently expressed Na+ channels, and a concentration of 30 μM

*Correspondence to: Chenhong Li; Email: lichhhust@yahoo.com
Submitted: 08/02/12; Revised: 09/11/12; Accepted: 09/12/12
http://dx.doi.org/10.4161/chan.22209
virtually eliminated all current activity. These data show that the skeletal muscle sodium channel isoform is blocked as potently as the native neuronal sodium channels in DRG neurons.  

Discussion

Hyperpolarization-activated cation nonselective cyclic nucleotide-gated (HCN) channel currents were first functionally described as the “funny” current $I_h$ in rabbit sinoatrial node and the “queer” current $I_q$ in hippocampal pyramidal neurons. Subsequently, it was also demonstrated as $I_h$ in a variety of peripheral sensory nerve preparations, such as dorsal root ganglia (DRG), rat and rabbit nodose ganglia and guinea-pig myenteric neurons. These excitatory currents push a cell toward the threshold of activation for action potentials, therefore play an important role in the modulation of heart rate and neuronal activities. Altered HCN currents in injured tissues may be an important mechanism underlying neuropathic pain, and drugs that modulate these currents may offer new therapeutic options.

Materials and Methods

Animals. All experiments performed on animals were performed under protocols approved by the Animal Care and Use Committee of our university in compliance with the Guide for the Care and Use of Laboratory Animals provided by Province Disease Control center of Hubei. Male Wistar rats weighing 100 g were used in all experiments. The rats were provided by Province Disease Control center of Hubei and housed in separated cages with free access to water and food. The room temperature was kept at 25°C and under a normal light-dark cycle.

Preparation of Dorsal root ganglion cells. DRG neurons were isolated from Wistar rats (200 g) that were killed by cervical dislocation and subsequent decapitation. DRG neurons were prepared as previously described. Briefly, all DRG contained in the lumbar 5–6 region were minced for 6 min by microscissors after dissection, then subjected to collagenase (2.5 mg/mL, type I, invitrogen) and trypsin (1.25 mg/mL, invitrogen) for 15 min at 37 degree. The enzymatic reaction was stopped when most cells were smooth and round. The reaction was stopped by washing the cells with DMEM containing 10% fetal bovine serum (FBS). Cells were collected by centrifugation at 1000 rpm for 6 min and then 200 uL fresh DMEM supplemented with 10% FBS was added to the cells. The cells were placed on poly-d-lysine (0.1 mg/mL, sigma)-treated glass coverslips contained within the cell culture dish and kept in 5% CO₂ incubator at 37 degrees. Patch clamp experiments were performed after 2–3 hours.

Cell culture and transfection. Human embryonic kidney cells (HEK293) were cultured in DMEM containing 10% FBS, with a stable temperature of 37 degree and 5% CO₂. pcDNA3.1-Nav1.4 and pcDNA3.1-HCN1 were co-transfected into HEK293 cells by Lipofection.

Whole-cell recording. Na⁺ current was measured by whole-cell patch clamp recording. Recordings were made with Axopatch 700B amplifier controlled by pClamp 10.0 (Molecular Devices, Inc., Sunnyvale, CA).

Figure 1. Na⁺ currents recorded in DRG neurons were identified by lidocaine, a selective blocker of sodium currents. (A) showed the Na⁺ currents recorded in a DRG neuron. Lidocaine almost completely inhibited the current, and the current could be recovered partially by washout. (B) Time course of current traces in the presence of Lidocaine (2 mM) recorded in response to 80 ms depolarizing pulses from -100 to -10 mV.
Sunnyvale, CA, USA). The output was filtered at 5 kHz and digitally sampled at 20 kHz using a DigitData 1440 Series interface (Molecular Devices). 80% of the series resistance was compensated by the analog circuitry. Data were recorded 10 min after whole-cell was established. Recordings were made at 25°C. Patch electrodes were fabricated with a PIP5 puller (HEKA, Germany) and the tip was heat-polished to a final tip resistance of 3–4 MΩ. Solution for sodium currents recording in DRG cells, the pipette contained (mM): CsF 140, NaCl 10, MgCl₂ 2, CaCl₂ 0.1, EGTA 1 and HEPES 10, pH was adjusted to 7.2 with CsOH. The bath

**Figure 2.** Inhibition of Na⁺ currents in DRG neurons by ZD7288. (A₁-A₄) Traces were recorded in the presence and absence of different concentrations of ZD7288 and after washout of ZD7288. Time course of peak current amplitudes in the presence of different concentrations [100, 30, 10 and 1 (μM)] of ZD7288 recorded in response to 80 ms depolarizing pulses from -100 to -10 mV. NOTE: Missing legend for Figure B₁-B₄.
solution contained (mM): NaCl 35, Choline chloride 105, CaCl$_2$ 1, MgCl$_2$ 1, CdCl$_2$ 0.1, TEA-Cl 20, HEPES 10, glucose 10. PH was adjusted to 7.4 with NaOH. For HCN channel currents recording in DRG cells, the pipette solution contained (mM): KCl 135, MgCl$_2$ 10, CaCl$_2$ 0.1, EGTA 1, HEPES 10, PH was adjusted to 7.2 with KOH. The bath solution contained (mM): NaCl 140, KCl 5, MgCl$_2$ 1, HEPES 10, CaCl$_2$ 2, D-glucose 10, PH was adjusted to 7.4 with NaOH. Solution for HEK293 patch, the pipette contained (mM): CsF 140, NaCl 10, EGTA 5 and HEPES 10. PH was adjusted to 7.2 with CsOH. Bath solution contained (mM): NaCl 140, glucose 5, MgCl$_2$ 1, KCl 3, CaCl$_2$ 1, HEPES 10. PH was adjusted to 7.4 with NaOH. All reagents were purchased from Sigma, otherwise indicated.

**Data analysis.** Voltage-clamp experimental data were analyzed using clampfit (V 10.0, molecular Devices), sigmaplot 10.0 and GraphPad prism 4. Dose response curves were fitted with the Hill equation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

This research was supported by the National Natural Science foundation of China (grant # 30972848 and 81271234) and special funds of basic research operating expenses for universities of China. We would like to thank Professor Ding (HUST) for his providing plasmids.
References

1. Brown HF, DiFrancesco D, Noble SJ. How does adrenaline accelerate the heart? Nature 1979; 280:235-6; PMID:450140; http://dx.doi.org/10.1038/280235a0.

2. Marshall PW, Rouse W, Briggs I, Hargreaves RB, Mills SD, McLoughlin BJ. ICI D7288, a novel sinoatrial node modulator. J Cardiovasc Pharmacol 1993; 21:902-6; PMID:7687714; http://dx.doi.org/10.1097/00005123-199306000-00008.

3. Luo L, Chang L, Brown SM, Ao H, Lee DH, Figuera ES, et al. Role of peripheral hyperpolarization-activated cyclic nucleotide-modulated channel pacemaker channels in acute and chronic pain models in the rat. Neuroscience 2007; 144:1477-85; PMID:17196750; http://dx.doi.org/10.1016/j.neuroscience.2006.10.048.

4. Li J, McRoberts JA, Nie J, Ennes HS, Mayer EA. Electrophysiological characterization of N-methyl-D-aspartate receptors in rat dorsal root ganglia neurons. Pain 2004; 109:443-52; PMID:15157705; http://dx.doi.org/10.1016/j.pain.2004.02.021.

5. Fan N, Sikand P, Donnelly DF, Ma C, Lamotte RH. Increased Na+ and K+ currents in small mouse dorsal root ganglion neurons after ganglion compression. J Neurophysiol 2011; 106:2111-8; PMID:21525373; http://dx.doi.org/10.1152/jn.00665.2011.

6. Brown HF, DiFrancesco D, Noble SJ. How does adrenaline accelerate the heart? Nature 1979; 280:235-6; PMID:450140; http://dx.doi.org/10.1038/280235a0.

7. Halliwell JV, Adams PR. Voltage-clamp analysis of muscarinic excitation in hippocampal neurons. Brain Res 1982; 250:71-92; PMID:6128061; http://dx.doi.org/10.1016/0006-8993(82)90954-4.

8. Maher MP, Wu NT, Guo HQ, Dubin AE, Chaplan SR, Wickenden AD. HCN channels as targets for drug discovery. Comb Chem High Throughput Screen 2009; 12:64-72; PMID:19149492; http://dx.doi.org/10.2174/138620709787048028.

9. Bucchi A, Barbieri A, Baruscotti M, DiFrancesco D. Heart rate reduction via selective ‘funny’ channel blockers. Curr Opin Pharmacol 2007; 7:208-13; PMID:17267284; http://dx.doi.org/10.1016/j.coph.2006.09.005.

10. Baruscotti M, Bucchi A, DiFrancesco D. Physiology and pharmacology of the cardiac pacemaker (“funny”) current. Pharmacol Ther 2005; 107:59-79; PMID:15963351; http://dx.doi.org/10.1016/j.pharmthera.2005.01.005.

11. Felix R, Sandoval A, Sánchez D, Gómora JC, De la Vega-Beltrán JL, Treviso CL, et al. ZD7288 inhibits low-threshold Ca(2+) channel activity and regulates sperm function. Biochem Biophys Res Commun 2003; 311:187-92; PMID:14575712; http://dx.doi.org/10.1016/j.bbrc.2003.09.197.

12. Sánchez-Alonso JL, Halliwell JV, Colino A. ZD 7288 inhibits T-type calcium current in rat hippocampal pyramidal cells. Neurosci Lett 2008; 439:275-80; PMID:18554748; http://dx.doi.org/10.1016/j.neulet.2008.03.016.