Original Research

Capsazepine concentration dependently inhibits currents in HEK 293 cells mediated by human hyperpolarization-activated cyclic nucleotide-gated 2 and 4 channels

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Abstract

Recent studies indicate that blockade of currents (I_h) mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (particularly HCN1) may partly account for the antinociceptive effects of capsazepine (CPZ). Unfortunately, determining whether capsazepine is a selective HCN channel blocker and determining its adverse effects when it is used for the treatment of neuropathic pain, have been thus far understudied. In this study, we aimed to elucidate the effects of capsazepine on human HCN2 (hHCN2) and HCN4 (hHCN4) channels in HEK293 cells. The vectors that expressed hHCN2 and hHCN4 cDNA were constructed and transfected into HEK293 cells. Enhanced green fluorescent protein (EGFP) fluorescence and the reverse transcription polymerase chain reaction (RT-PCR) were used to confirm the successful transfection of the vectors. After G418 (neomycin) screening, cell lines that expressed hHCN2 and hHCN4 were obtained. The whole-cell voltage-clamp technique was used to determine the currents from hHCN2 and hHCN4 channels, which were perfused with five concentrations (0.1 μM, 1 μM, 5 μM, 10 μM and 50 μM) of capsazepine. The results showed that capsazepine at the range from 0.1 to 50 μM markedly inhibited hHCN2 and hHCN4 currents in a concentration-dependent manner, with most inhibition achieved at a concentration of 10 μM of capsazepine. When compared with the control group, a $V_{0.5}$ for the hHCN2 and hHCN4 channel showed that 10 μM capsazepine significantly shifted the membrane potential towards hyperpolarization. The present results indicate that capsazepine is not a selective HCN1 channel blocker and that it may have adverse effects when used to treat neuropathic pain.

Keywords: Capsazepine, human hyperpolarization-activated cyclic nucleotide-gated channel, patch clamp

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Introduction

Cardiac and neuronal pacemaker activity and the determination of resting membrane potentials are mainly modulated by the currents from hyperpolarization activated cyclic nucleotide gated (HCN) channels.[1–3] Structurally, the four members of the HCN channel family (HCN1–4) belong to the six transmembrane ion channel superfamily.[4,5] HCN1 is mainly located in the peripheral nervous system and fine sensory brain regions. The expression of HCN3 is almost nonexistent in the nervous system and heart. In contrast, because they are abundant in heart, HCN2 is involved in the formation of the heart rhythm, and HCN4 possesses a dominating position in cardiac transduction tissues (such as the sinus node and Purkinje fibers).[6] Following the introduction of the concept of ''ion channel disease'', the HCN channel inhibitor capsazepine (CPZ)[7,8] has been reported to effectively treat neuropathic pain, and the HCN channel inhibitor ivabradine[9] has been reported to effectively treat cardiac arrhythmias. However, it is unclear whether capsazepine is a selective HCN1 channel blocker or whether it also blocks HCN2 or HCN4 channels. Recently, Demontis *et al.*[10] reported interesting effects of selective HCN1 channels inhibition by ivabradine in mouse rod photoreceptors. It is well established that hormones and neurotransmitters can modulate HCN activity via G-protein pathways that modulate cAMP concentrations.[1] Thus, we hypothesized that capsazepine may modulate HCN2 and/or HCN4 channels via cAMP-dependent pathways and designed this study to elucidate the effects of capsazepine on hHCN2 and hHCN4 channels in HEK293 cells.

Materials and methods

Vector and cell culture

Full-length hHCN2 and hHCN4 cDNAs were subcloned into pIRES2-EGFP vectors (Life Technologies Corporation, Shanghai, China). HEK293 cells were obtained from the American Tissue Culture Collection and maintained in Dulbecco's modified Eagle's medium (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (Hyclone), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C, 5% CO_2, and 95% air.

Transfection and reverse transcription polymerase chain reaction (RT-PCR)

The vectors that expressed human HCN2/4 and EGFP were transfected into HEK293 cells using FuGENE HD transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturers' specifications (transfectant/DNA ratio, 5/2 v/w). This was completed when six-well plates reached 80–90% confluency. EGFP expression was determined by inverted fluorescence microscopy 36 h after transfection (Olympus, Tokyo, Japan).

Total RNA of hHCN2 and hHCN4 was extracted from the transfected HEK293 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed as previously described.[11] Specific primers for human HCN2 (forward primer: 5′-CGCCTGATC CGCTACACAT-3′, reverse primer: 5′-AGTGCGAAGGAGTACAGTTCACT-3′), HCN4 (forward primer: 5′-CCCGCCTCATTCGATAT ATTCAC-3′, reverse primer: 5′-GAGCGCGTAGGAGTA CTGCTTC-3′) were used to detect the expression of these two genes. PCRs (20 μl final volume) were performed using an Icycler Thermal Cycler (Bio-Rad, Hercules, CA, USA) and the following amplification steps: 95°C for 5 min, 95°C for 35 s, 59°C for 45 s, 72°C for 45 s (30 cycles) and 72°C for 10 min. Amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. PCR product bands were visualized by ultraviolet light.

Establishment of stable hHCN cell lines

Forty-eight hours after transfection, 0.6 mg/ml G418 was added to the cell medium for selection. After four weeks, resistant cell clones were selected and checked for the presence of HCN currents by patch-clamp recordings. For prolonged storage, cells in complete fetal bovine serum with 10% (v/v) dimethylsulfoxide (DMSO) were kept in liquid nitrogen.

Whole cell patch-clamp recording

Heterologously expressed HCN channels were measured at room temperature with the whole-cell voltage-clamp technique by a patch-clamp amplifier (HUST-IBB, PC-2B; Huazhong University of Science and Technology, Wuhan, China) and then stored using the IBBClamp computer software as previously described.[12] Patch pipettes were pulled from thin-walled borosilicate glass on a vertical puller (PP-830; Narishige, Japan). The resistance between the recording electrode filled with the pipette solution and the reference electrode was 2–5 MΩ. Capacity transients were cancelled as much as possible, and voltage errors were reduced by 80–90% by series resistance compensation. Leakage currents were digitally subtracted. We corrected for the liquid junction potential throughout all experiments. Currents mediated by HCN channels were recorded 3 min after the establishment of the whole cell configuration. All experiments were performed at room temperature.

The effects of capsazepine were determined with a repetitive stimulation protocol.[13] Whole-cell, HCN-encoded currents were evoked in response to hyperpolarizing voltage steps between −50 and −140 mV from a holding potential of −40 mV.

Drugs and solutions

The extracellular solution was composed of 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES. It was adjusted to a pH of 7.4 with KOH. The intracellular solution contained 130 mM KCl, 10 mM NaCl, 0.5 mM $MgCl_2$, 1 mM EGTA and 5 mM HEPES. Its pH was adjusted with KOH to 7.4.

All drugs were purchased from Sigma (St Louis, MO, USA), with the exception of *N*-[2-(4-Chlorophenyl)ethyl]-1,3,4,5-tetrahydro-7,8-dihydroxy-2H-2-benzazepine-2-carbothioamide (capsazepine) (Tocris Cookson, Bristol, UK). Capsazepine and ZD7288 were dissolved in DMSO.

Data analysis

All continuous data are expressed as the mean ± the standard error of the mean (SEM). These data were compared by Student's paired or unpaired *t*-tests, as appropriate, to evaluate the statistical significance of differences between the two group. Analysis of variance (ANOVA) was used to compare multiple groups. Concentration-effect data were fitted to the Hill equation: $y = 1/(1 + (IC_{50}/x)\ nH)$, where the IC_{50} is the half-block concentration, x is drug concentration, and *nH* is the Hill coefficient. Steady-state activation curves were fitted to the Boltzmann equation: $I/Imax = [1 + \exp((V_m - V_{0.5})/k)]^{-1}$ (V_m : the membrane potential; $V_{0.5}$: the potential of half-maximal activation; k: the slope factor). All data were analyzed and fitted by Clampfit (Axon Instruments, Foster City, CA, USA) and SigmaPlot (SPSS Inc., Chicago, IL, USA) software. Statistical significance was defined as $P < 0.05$ and evaluated using two-sided tests.

Results

Human HCN2/4 expression in HEK293 cells

Human HCN2/4 vectors and the EGFP vector were successfully transfected into HEK293 cells (Figure 1(a–c)). Thereby, heterologous expression of hHCN2 and hHCN4 channels was established.

For RT-PCR analysis, expression of PCR products from the HEK293 cells transfected with hHCN2/4 and EGFP was measured and compared with that from the untransfected ones. DNA ladders were used as DNA molecular weight markers. We could acquire the amplified PCR products only from the cells transfected cells with hHCN2/4, and

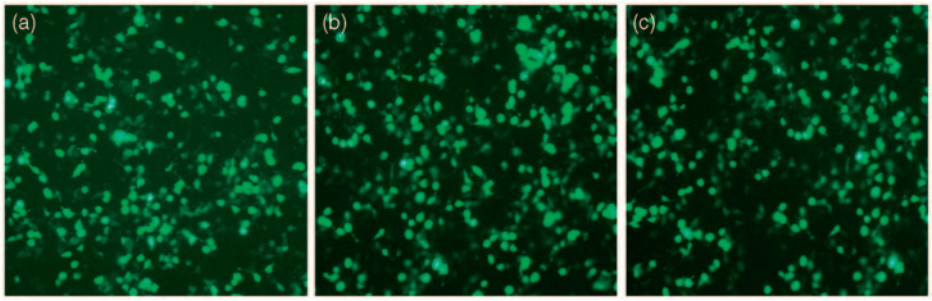

Figure 1 Human HCN2/4 expression in HEK293 cells. EGFP fluorescence is shown after transfection for 36 h. The cells were transfected with pIRES2-HCN2-EGFP vector (a), pIRES2-HCN4-EGFP vector (b), or pIRES2-EGFP vector (c)

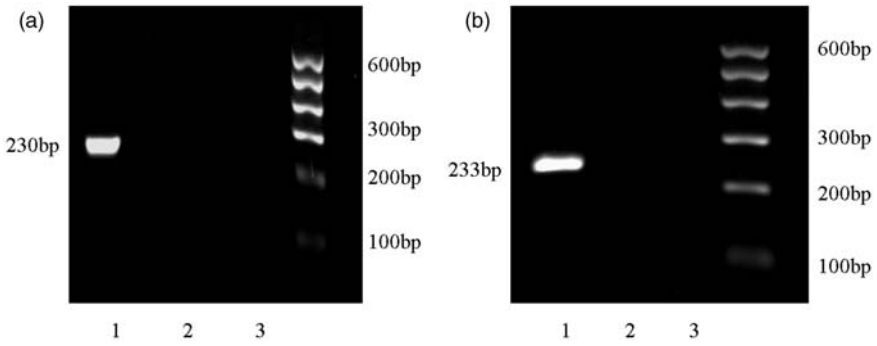

Figure 2 RT-PCR analysis of HEK293 cells transfected with hHCN2 and hHCN4 channels. (a) The PCR products of hHCN2 and hHCN4 pointed at 230 bp and 233 bp, respectively. (a) Cells transfected with hHCN2 (lane 1) and (b) expression of hHCN4 (lane 1). Other lanes presented show EGFP-vector expression (lane 2 in (a) and (b)) or untransfected cells (lane 3 in (a) and (b))

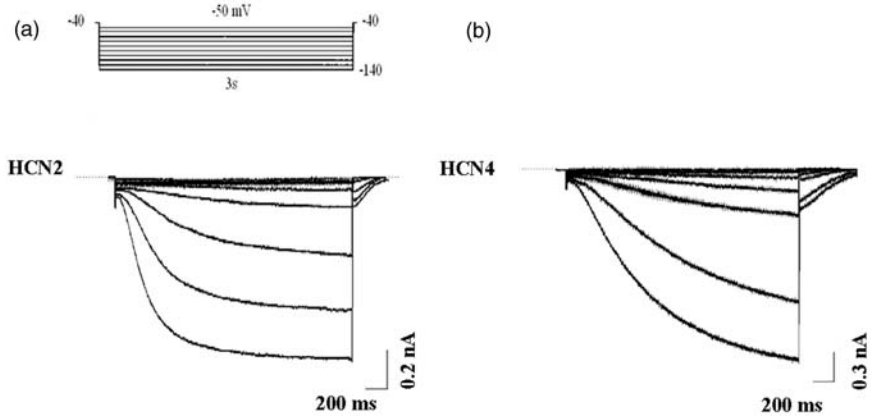

Figure 3 Inward currents mediated by hHCN2 or hHCN4 channels in HEK293 cells. (a) Example hHCN2 channel currents. Upper: voltage protocol. Cells were clamped from a holding potential of −40 mV to various voltages (−140 to −50 mV in 10 mV increments) for 3 s. Lower: current traces of a cell expressing hHCN2. (b) Example hHCN4 channel currents. Upper: voltage protocol. Lower: the hHCN4 current traces

not cells transfected with EGFP or untransfected cells, as shown in Figure 2. The 230 bp band was consistent with HCN2 cDNA fragments, whereas the 233 bp band corresponded to HCN4 cDNA fragments. All RT-PCR experiments were conducted in triplicate ($n = 3$) as previously described.[14]

Identification of heterologous expression hHCN2/4 channels currents

A family of inward currents from HEK293 cells expressing hHCN2 or hHCN4 channels are shown in Figure 3. In whole-cell voltage patch-clamp mode, hyperpolarizing voltage steps induced inward currents in hHCN2-(Figure 3(a), $n = 7$) and hHCN4-expressing (Figure 3(b), $n = 8$) cells. HEK293 cells transfected with EGFP reporter genes showed no hyperpolarization inward currents (not shown). These results are consistent with those of a previous study.[15]

The pharmacological properties of the hHCN2 and hHCN4 channels

Cs^+ and ZD7288 were effective inhibitors of hHCN2 and hHCN4-mediated currents, and their blocking effects on the two channels were clear. HEK293 cells expressing hHCN2

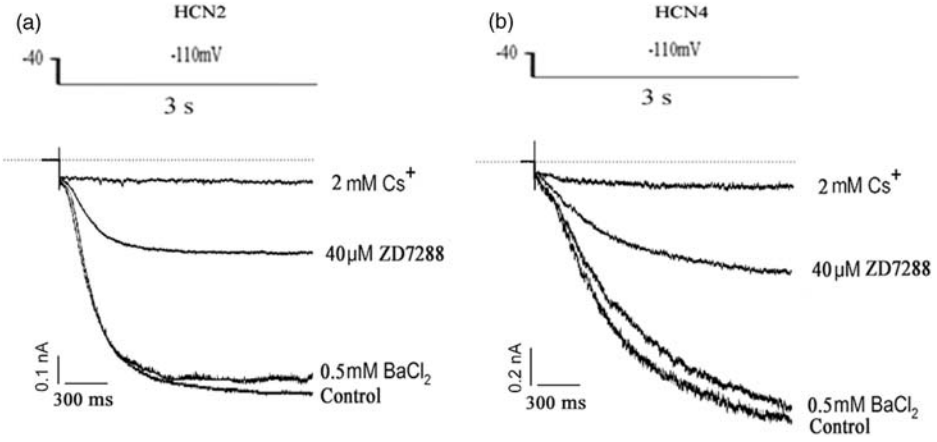

Figure 4 Cs^+, ZD7288, and $BaCl_2$ effectively inhibited hHCN2 and hHCN4 currents. hHCN2 (a) and hHCN4 (b) currents were evoked from a holding potential of −40 mV with a step to −110 mV for 3 s. hHCN2 (a) and hHCN4 (b) current traces under the control condition and with 2 mM CsCl. hHCN2 (a) and hHCN4 (b) current traces in the control condition and with 40 μM ZD7288. hHCN2 (a) and hHCN4 (b) current traces in the control condition and with 0.5 mM of extracellular $BaCl_2$

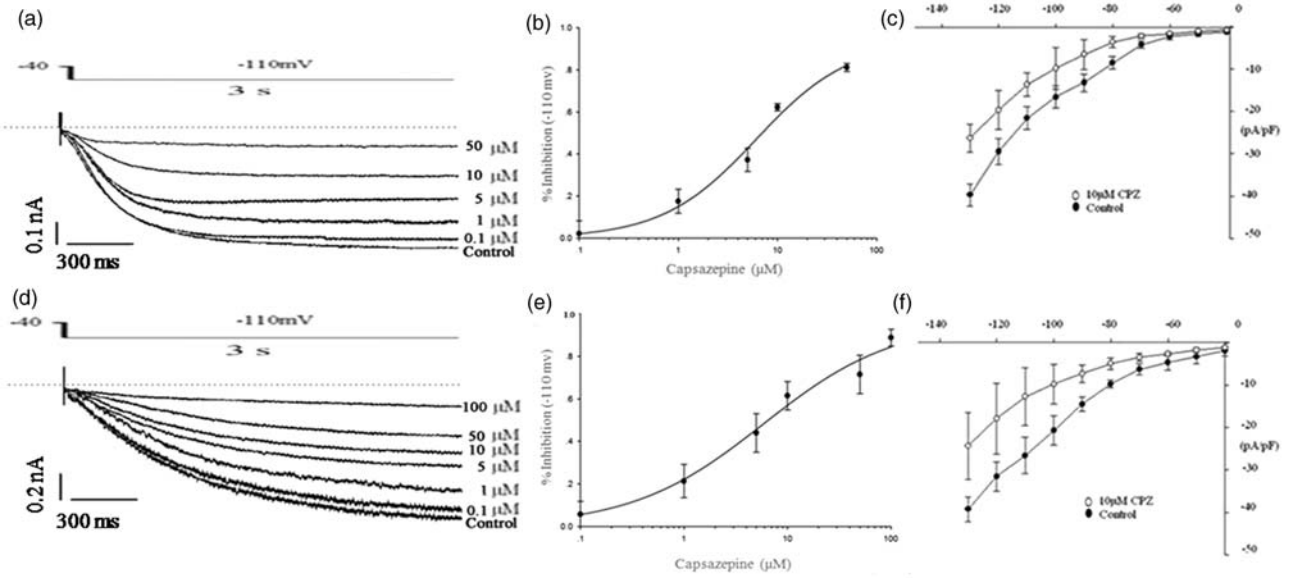

Figure 5 Capsazepine concentration-dependently inhibited the hHCN2 and hHCN4 channels. Current traces of stably expressed HCN2 (a) and HCN4 (d) in the presence of increasing concentrations of capsazepine. Currents were evoked by stepping from −40 to −110 mV. Concentration-response relationships for the inhibition of HCN2 (b) and HCN4 (e) currents by capsazepine. The solid lines are the fits to the Hill equation with the following parameters: HCN2, $IC_{50} = 6.1 \pm 0.8$ μM; HCN4, $IC_{50} = 5.8 \pm 0.5$ μM. I–V curve for HCN2 (c) and HCN4 (f) before and after application of 10 μM capsazepine. Each data point represented the mean ± the S.E.M ($n = 10$)

or hHCN4 channels activated by a voltage step to −110 mV were blocked by 2 mM Cs^+ and 40 μM ZD7288 as shown in Figure 4. On average, 2 mM Cs^+ blocked 95 ± 4% (Figure 4(a), $n = 6$) of the hHCN2-mediated currents, whereas ZD7288 (40 μM) blocked 68 ± 3% (Figure 4(a), $n = 4$). In contrast, 2 mM Cs^+ blocked 92 ± 3% (Figure 4(b), $n = 4$) of the hHCN4-mediated currents, whereas ZD7288 (40 μM) blocked 65 ± 5% (Figure 4(b), $n = 5$). Neither channel current was sensitive to 0.5 mM Ba^{2+} ($n = 4$ for hHCN2; $n = 5$ for hHCN4). These results support the typically reported properties of hHCN2 and hHCN4 channels expressed in HEK cells.[13,15,16]

Effects of capsazepine on the hHCN2 channel

As shown in Figure 5(a), capsazepine at 0.1 μM, 1 μM, 5 μM, 10 μM, and 50 μM markedly inhibited hHCN2 currents by 2.1 ± 4.0% ($n = 7$, $P > 0.05$), 17.5 ± 3.6% ($n = 8$, $P < 0.05$), 37.2 ± 3.3% ($n = 6$, $P < 0.05$), 62.2 ± 5.6% ($n = 10$, $P < 0.05$), and 81.2 ± 0.9% ($n = 7$, $P < 0.05$), respectively. When these data were fitted with the Hill equation, the fitting curves revealed an $IC_{50} = 6.1 \pm 0.8$ μM (Figure 5(b)). The use of 10 μM capsazepine produced a significant upward shift in the I–V curve (Figure 5(c)). The I–V curve for the hHCN2 channel indicated that the current densities were reduced from −8.54 ± 1.45 pA/pF to −3.62 ± 1.31 pA/pF at −90 mV

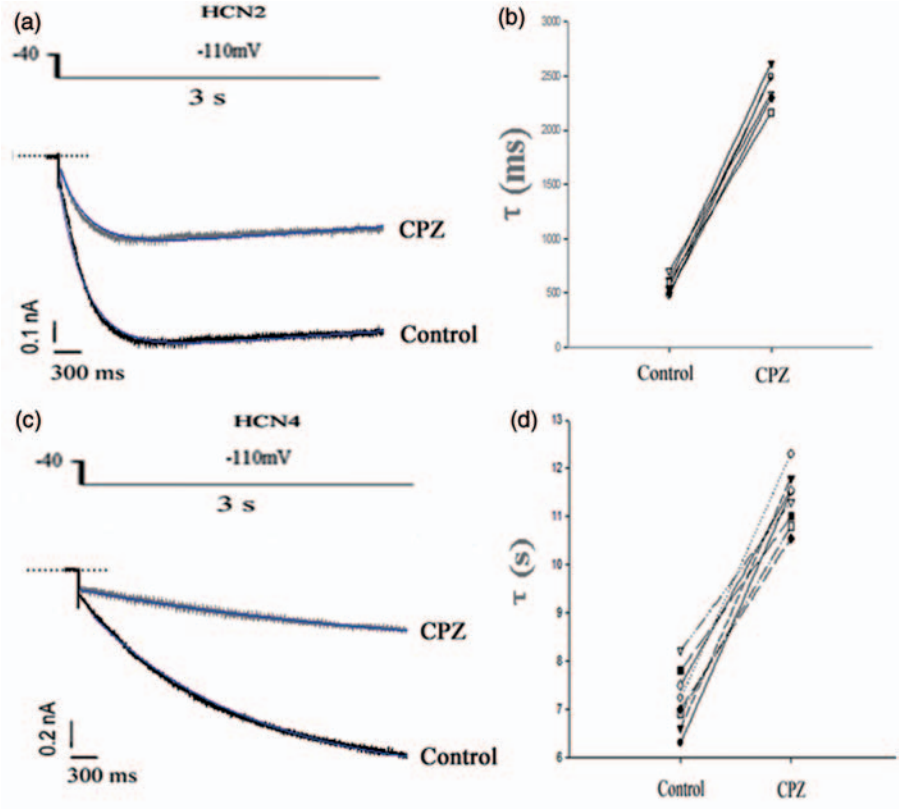

Figure 6 Activation kinetics of hHCN channels. The control current obtained at −110 mV (dark blue trace) compared with currents taken after perfusion of 10 μM capsazepine (light blue trace) for hHCN2 (a) and hHCN4 (c) channels. Blue lines are a single exponential fit to hHCN channel currents activation phase. Activation time constants (τ) of hHCN2 (b; $n = 7$) and hHCN4 (d; $n = 8$) at −110 mV in the control condition compared with one obtained after administration of 10 μM capsazepine. Lines connect the τ values in both conditions for a cell. (A color version of this figure is available in the online journal)

and from -29.46 ± 3.1 pA/pF to -19.67 ± 4.6 pA/pF at −130 mV ($n = 10$, all $P < 0.05$).

Effects of capsazepine on the hHCN4 channel

As shown in Figure 5(d), at a test potential of −110 mV, application of capsazepine at 0.1, 1, 5, 10, 50, and 100 μM inhibited hHCN4 currents by $5.7 \pm 6.2\%$ ($n = 8$, $P > 0.05$), $21.3 \pm 7.8\%$ ($n = 6$, $P < 0.05$), $44 \pm 9\%$ ($n = 9$, $P < 0.05$), $61.5 \pm 6.7\%$ ($n = 7$, $P < 0.05$), $71.5 \pm 9\%$ ($n = 5$, $P < 0.05$), and $88.9 \pm 4.0\%$ ($n = 10$, $P < 0.05$), respectively. The IC_{50} was approximately 5.8 ± 0.5 μM (Figure 5(e)). Population data showed that 10 μM capsazepine produced a significant upward shift in the I–V curve (Figure 5(f)), and the current density was decreased between −90 or even less (e.g. at −90 mV: -9.72 ± 0.91 pA/pF vs. -4.86 ± 1.37 pA/pF and at −130 mV: -31.51 ± 3.44 pA/pF vs. -17.86 ± 4.1 pA/pF) ($n = 10$, all $P < 0.05$).

Activation kinetics of hHCN2 and hHCN4 channels

The voltage dependencies of the activation time constants are shown in Figure 6. The hHCN2 current (Figures 3–6) activated much more quickly than the hHCN4 current (Figures 3–6). Current activation at −110 mV was fitted with a single exponential function with a $\tau = 552 \pm 78$ ms under control conditions and with a $\tau = 2358 \pm 202$ ms with 10 μM capsazepine for hHCN2 ($n = 7$, $P < 0.05$; Figure 6(a, b)). In contrast, for hHCN4, current activation at −110 mV was fitted with a single exponential function with a $\tau = 7.2 \pm 1$ s under control conditions and with a $\tau = 11.5 \pm 1.2$ s in the presence of 10 μM capsazepine ($n = 8$, $P < 0.05$; Figure 6(c, d)).

Effects of capsazepine on voltage-dependent activation of the hHCN2 and hHCN4 channels

The activation of HCN channels was fitted with a Boltzmann function. For the hHCN2 channel, as shown in Figure 7, 10 μM capsazepine shifted the $V_{0.5}$ potential in the hyperpolarizing direction (control, -95.41 ± 2.0 mV; capsazepine, -102.1 ± 1.47 mV; $n = 10$, $P < 0.05$). In the case of the hHCN4 channel, capsazepine also shifted the $V_{0.5}$ potential in the hyperpolarizing direction (control, -98.83 ± 1.62 mV; capsazepine, -114.8 ± 2.43 mV; $n = 10$, $P < 0.05$).

Discussion

To our knowledge, this is the first report to show that capsazepine engenders significant, concentration-dependent inhibition of currents from hHCN2 and hHCN4 channels and affects voltage-gated activation of hHCN2 and hHCN4 channels.

Capsazepine was discovered by a research group at the Sandoz Institute for medical research. Its synthesis and chemical properties were published in 1994. It was derived from modification of the chemical backbone of capsaicin.[17]

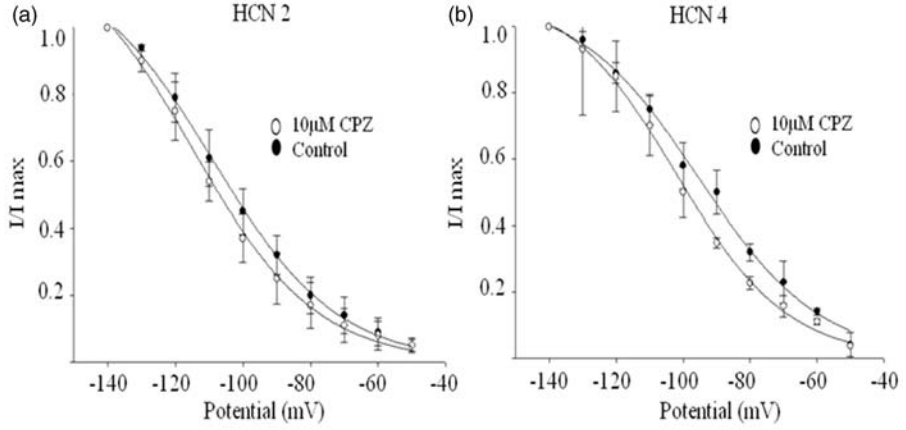

Figure 7 Capsazepine shifted the activation curves of the HCN channels. The activation curves were fitted with a Boltzmann function. The use of 10 μM capsazepine produced a 6.6 mV left shift of the $V_{0.5}$ activation voltage for the HCN2 and 16 mV shift of the $V_{0.5}$ for the HCN4 activation voltage

Capsazepine blocks the painful sensation of heat caused by capsaicin (the active ingredient of chili pepper) activating the TRPV1 (transient receptor potential vanilloid type 1) ion channel. It is therefore considered to be a capsaicin antagonist. The TRPV1 channel functions as a pain and temperature sensor in mammals. Capsazepine blocks only the activation of TRPV1 channels by chemicals, but not by other painful stimuli such as heat. Depending on the pharmacological assay, its half maximal inhibitory concentration (IC50) is in the nanomolar to low micromolar range. In addition to its effects on TRPV1 channels, it inhibits cold-activated TRPM8 channels,[18] voltage-activated calcium channels,[19] and nicotinic acetylcholine receptors.[20] It mainly serves as a tool to study the TRPV1 ion channel.[21] Recently, some researchers have demonstrated that HCN channels exhibit structural similarities to TRPV1[3,22] and participate in neuropathic pain.[23] Thus, it has been proposed that blockade of HCN channels (particularly HCN1) may be a component of antinociception induced by capsazepine.[7,24] Unfortunately, few reports have described whether capsazepine is a selective HCN channel blocker, or whether it has adverse effects when used in the treatment of neuropathic pain. In our experiments, hHCN2 and hHCN4 cDNA was transfected into HEK293 cells. Moreover, time- and voltage-dependent I_h-like inward currents were found in HEK293 cells transfected with hHCN2 or hHCN4. The half-activation voltage ($V_{1/2}$) and activation time constants (τ)(−110 mV) for hHCN2 and hHCN4 channels, resemble the values reported by Stieber *et al.*[16,25] and Ludwig *et al.*[13] The nonselective effects of capsazepine on hHCN2 and hHCN4 channels are the same as those of ivabradine (a specific inhibitor of I_f channels) on HCN1 channels.[10] The native I_f current, mainly constituted by HCN2 and HCN4 channels in cardiac transduction systems, is very important to cardiac rhythm initiation and frequency modulation. Thus, it has been proposed that blockade of I_f currents may lower heart rates and reduce the firing frequency of sinus node cells.[6,26,27] A number of studies have reported that ivabradine reduces heart rates by inhibiting I_f currents, and it is used to treat angina.[9,28–31] Considering that HCN2 and HCN4 are widely expressed in the heart and constitute the major components of I_f currents, we assumed that capsazepine may inhibit I_f currents, and lower heart rates or induce bradyarrhythmia.

As mentioned above, TRPV1 exhibits structural similarities to HCN channels. In addition, both TRPV1 and HCN channels are expressed in heart[32] and are associated with cardiovascular disease.[33] Phillips *et al.*[34] reported capsazepine interacts with TRPV1 through binding to the S2–S4 region of the channel, which was confirmed by mutation of the amino acids within the S2–S4 region. Gill *et al.*[7] speculated that capsazepine inhibits hHCN1 via interactions with the extracellular domain between S1 and S2. Moreover, as both TRPV1[35] and HCN channels[6] are sensitive to pH, capsazepine may interact with HCN channels through modulating extracellular pH levels. In this study, we investigated the effects of capsazepine on currents mediated by hHCN2 and hHCN4. Because of the complex structure of HCN channels, which include a cyclic nucleotide-binding domain, we did not mutate the S1–S2 and S2–S4 position of their structures. However, future studies should elucidate the precise mechanisms underlying inhibition of HCN channel-mediated currents by capsazepine.

In summary, capsazepine can inhibit the currents from hHCN2/4 channels, suggesting that it is not a selective HCN1 channel blocker. This finding may be of great relevance for other neuronal and cardiovascular effects of capsazepine and its analogues. Because HCN1, HCN2, and HCN4 channels are ubiquitously expressed in the nervous system and heart, and participate in diverse functions, therefore propose capsazepine may have adverse effects when it is used for treating neuropathic pain. It may be necessary to develop agents that can selectively block the HCN1, HCN2, or HCN4 channels. Moreover, the effects of capsazepine on cardiac rhythms should be investigated in further studies.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript. G-FZ conducted the patch-clamp experiments and wrote the manuscript; M-HL, J-XZ, BL, and Z-MW conducted the molecular biological

experiments; QW analyzed the data; HX revised the whole manuscript; S-LC designed the experiment and reviewed the whole manuscript.

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