Novel Role of KT5720 on Regulating Hyperpolarization-Activated Cyclic Nucleotide-Gated Channel Activity and Dorsal Root Ganglion Neuron Excitability

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Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels are expressed in dorsal root ganglion (DRG) neurons, which are involved in diverse mechanisms that regulate DRG functions. Protein kinase A (PKA) is an essential kinase that plays a key role in almost all types of cells; it regulates the ion channel activity, the intracellular Ca^{2+} concentration, as well as modulates cellular signals transduction. Nevertheless, the effect of PKA inhibition on the HCN channel activity in DRG neuron remains to be elucidated. Here we investigated the impact of PKA inhibition on the HCN channel activity and DRG neurons excitability. Our patch-clamp experiments both under whole-cell and single-channel conditions demonstrated that PKA inhibition with KT5720, a cell membrane permeable PKA-specific inhibitor, significantly attenuated HCN channel currents. Current clamp recording on freshly isolated DRG neurons showed KT5720 reduced overshoot amplitude and enhanced the threshold of the action potential. Moreover, our live-cell Ca^{2+} imaging experiments illustrated KT5720 markedly reduced the intracellular Ca^{2+} level. Collectively, this is the first report that addresses KT5720 attenuates the HCN channel activity and intracellular Ca^{2+} , thus reducing DRG neurons excitability. Therefore, our data strongly suggest that PKA is a potential target for curing HCN and DRG neuron relevant diseases.

Introduction

HYPERPOLARIZATION-ACTIVATED cyclic nucleotide-gated (HCN) channels belong to the potassium channel superfamily. With a structure of 6 transmembrane domains S_1 – S_6 , within which, S_4 serves as a voltage sensor. In C-terminal, there is a cyclic nucleotide-binding domain. This unique structure renders the HCN channel the characteristics of being able to be regulated by both membrane voltage and intracellular cyclic adenosine monophosphate (cAMP).

HCN channels were extensively expressed in the heart, brain, and nerve systems (Brown *et al.*, 1979; Shi *et al.*, 1999; Demontis *et al.*, 2002; Bender *et al.*, 2003; Holderith *et al.*, 2003). Its expression patterns are roughly changed between the different parts of the body and species (Moosmang *et al.*, 1999; Monteggia *et al.*, 2000; Santoro *et al.*, 2000), this expression variety is probably implicated with different physiological functions. HCN channel encoded current I_h is involved in regulating a variety of neuron biophysical functions, including setting the resting membrane potential (Gauss and Seifert, 2000), controlling neurons excitability (Kase and Imoto, 2012), mediating action potentials (APs) (Hogan and Poroli, 2008), governing motor learning (Lewis *et al.*, 2011), regulating synaptic transmission, and dendritic integration as well (Atkinson and Williams, 2009). Thus, it is

not surprising that I_h plays an important role in both physiological and pathophysiological conditions. For example, the HCN channel plays a vital role in developing neocortex after birth (Battfeld *et al.*, 2012); In a hypertrophic heart, increased I_h prolongs the ventricular AP repolarization (Hofmann *et al.*, 2012); The HCN channel is a potential drug target for the treatment of epilepsy (Shah *et al.*, 2013); HCN2 channels play a central role in inflammatory and neuropathic pains, its mutation is potentially associated with generalized epilepsy with recessive inheritance as well (DiFrancesco *et al.*, 2011; Emery *et al.*, 2011).

To date, emerging experimental evidences indicate all four HCN channel subunits are expressed in murine dorsal root ganglion (DRG) neurons (Kouranova *et al.*, 2008), and previous studies have well documented that the HCN channel activity is regulated by intracellular cAMP (Almanza *et al.*, 2012). The cAMP/protein kinase A (PKA) is a critical pathway that controls a serial of cellular processes, recent reports showed regulating PKA has feedback on intracellular cAMP levels (Vandamme *et al.*, 2012). Hence, it is legitimate to postulate that PKA plays a role in regulating the HCN channel activity. However, currently available literatures provide controversial results. PKA inhibitor H-8 demonstrated significantly attenuated I_h in bull-frog sympathetic neurons (Tokimasa and Akasu, 1990), but the same

compound was identified to have no effect on HCN channel currents in canine purkinje fibers (Chang *et al.*, 1991). To address this issue, here, on freshly isolated rat DRG neurons, we chose KT5720, a membrane permeable selective PKA inhibitor, combined with cutting edge technologies, including whole-cell and single-channel patch-clamp recording, and live-cell calcium imaging. Our results demonstrated that KT5720 dramatically inhibited I_h in both whole-cell and single-channel conditions. Furthermore, we provided evidences that KT5720 decreased the intracellular calcium concentration in freshly isolated rat DRG neurons. Our data unveiled that PKA plays a key role in regulating the HCN channel activity and DRG neuron excitability, so it may represents a potential target in the treatment of HCN channel-related diseases.

Materials and Methods

Thirty-eight adult male Sprague-Dawley Rats with an average weight of 248.6 ± 13.9 g were used in this study. All the animal operation procedures were reviewed and approved by the Animal Care Committee of Huazhong University of Science & Technology; it also abided by the NIH guidelines.

DRG neurons isolation

Rats were euthanized with CO_2 , followed by neck dislocation. The back skin was incised, and then the backbone was quickly removed and put into a dish with oxygen saturated Dulbecco's Modified Eagle's Medium (DMEM) solution. After that, the backbone was cut into two parts along the midline, DRG was carefully taken out with a hairspring tweezer, the attached tissue was removed with an iris scissor, and the supernatant was removed by a clean straw. Then, the DRG tissue was completely cut into pieces with a spring scissor. The tissue was then put into a glass tube that contained enzymes, followed by gently mixing and percussing with a pipette. The tube was then put into a 37°C shaking bath for 15 min, wind and percussion several times every 5 min, and then phenylmethanesulfonyl fluoride was added into the suspension to terminate digestion. The suspension was then centrifugalized in 1000 r/min for 5 min, and then the supernatant was removed. Next, a 5 mL fresh DMEM was added into the tube and single DRG neurons were mixed well with a Pasteur pipette. The DRG neuron suspension was then dropped onto coverslips, which had been coated with poly-L-lysine. After that, these coverslips were placed in a 37°C CO_2 incubator for 30 min to let the cells adhere tightly, 2.5 mL of the DMEM was then added into each dish plus waiting for 1 h. DRG neurons obtained from these procedures were used for experiments.

Electrophysiological whole-cell patch-clamp recording

Whole-cell currents were recorded with Axopatch 200B, Digidata 1440A, and pClamp 10.2 software (Molecular Devices, Union city, CA), signals have been filtered with an eight-pole Bessel filter 900CT/9L8L (Frequency Devices, Ottawa, IL). Ten millimeter length borosilicate glasses (Sutter Instruments, Novato, CA) were used to prepare for the patch pipettes. The pipettes were pulled with a Narishige PP-830 Two-step puller, and then polished with a Micro Forge MF-

830 fire polisher (Narishige Group, Tokyo, Japan) to obtain a final resistance of 3–5 M Ω .

Electrophysiological single-channel patch-clamp recording

Single-channel recording was implemented in a cell-attached mode. Fire-polished borosilicate glass pipettes with a final resistance of 8–10 M Ω were chosen for recording, those seal resistance ≥ 10 G Ω were considered for data analyses, 10 μM ZD7288 was added to ensure that the recorded single-channel currents were I_h (data did not show). Only those rounds, shiny looking DRG neurons were chosen for experiments. All single-channel experiments were implemented at room temperature 25°C – 26°C .

Live-cell calcium imaging

For intracellular Ca^{2+} imaging experiments, 250 μL poly-L-lysine was added to each 35-mm-diameter glass bottom dish, incubated at 37°C , poly-L-lysine was put on the coverslip and incubated for 1 h, then dishes were aspirated and washed with 2 mL sterile water three times. Cell suspension was added into the dishes, kept at room temperature for 30 min to let the cells adhere to the bottom of the dishes. The Fura 2-AM solution was diluted with a bath solution to a final concentration of 2 μM . The supernatant was then removed from the dishes, 250 μL of the Fura 2-AM working solution was added into the dishes, and stored in darkness at room temperature for 30 min. The Fura 2-AM working solution was then removed and cells were washed with a bath solution 3 times. Ca^{2+} imaging at $\times 40$ oil objective was acquired with an OLYMPUS IX81 motorized inverted research microscope, the corresponding software was MetaFluor 7.7.2.0.

Solutions and drugs

The DRG tissue-containing solution has the following compositions (in mg/mL): DMEM 10 pH 7.4 with NaOH. The DRG digestion solution contains (in mg/mL) DMEM 10, type I collagenase 1, trypsin 0.5 pH 7.4 with NaOH. The whole-cell patch-clamp recording bath solution contains (in mM) NaCl 80, KCl 5, BaCl_2 2, NaH_2CO_3 26, CoCl_2 2, TEAC 40, CaCl_2 1, 4-AP 4, CdCl_2 0.2, D-Glucose 10 pH 7.4 with NaOH, 1 μM TTX was added to block irrelevant sodium channels. The whole-cell patch-clamp recording pipette solution contains (in mM) NaCl 5, K^+ -gluconate 135, ATP- Mg^{2+} 2, GTP- Na^+ 0.4, EGTA 0.5, HEPES 10 pH 7.3 with KOH. The single-channel patch-clamp recording bath solution contains (in mM) potassium gluconate 100, KCl 50, NaCl 4, CaCl_2 1, EGTA 5, HEPES 10, and glucose 10 pH 7.25 with KOH. The single-channel patch-clamp recording pipette solution contains (in mM) NaCl 140, KCl 5, CaCl_2 2, MgCl_2 1, HEPES 10 pH 7.4 with NaOH, plus 1 μM TTX, 10 mM TEA, 0.2 mM CdCl_2 , 10 μM picrotoxin was supplemented to block other voltage- and ligand-gated channels. All the compounds and chemicals were purchased from Sigma.

Data analysis and statistics

Clampfit 10.2 software (Molecular Devices, LLC, Sunnyvale, CA) was used for the patch-clamp electrophysiological data analyses. SigmaPlot 10.0 software (Systat software, Inc., Chicago, IL) and Igor Pro 5.0.1 (WaveMetrics, Inc., Lake

Oswego, OR) were used for statistical analysis and figure illustration. τ was fitted with a monoexponential equation. In step protocol, the currents were taken from the averaged last 100 ms recording. The single-channel current amplitudes were calculated from all points histograms, and the mean single-channel conductance values were calculated from the current-voltage curve. The results were summarized as means $\pm$ SEM from cell numbers n and animal numbers N . Data were compared with two-way ANOVA or the paired two-tailed student's t -test. p -value less than 0.05 was considered statistically significant.

Results

The selective PKA inhibitor KT5720 attenuates I_h in freshly isolated rat DRG neurons

The effect of PKA inhibition on DRG neuron I_h was investigated by the conventional whole-cell patch-clamp technique. At the holding potential of -40 mV, whole-cell I_h was elicited with a protocol that hyperpolarized from -60 to -140 mV in -10 mV interval, the hyperpolarize stimulation maintained for 2 s, then returned to the holding potential for 1 s. The cell capacitance was kept unchanged during the recording procedure. In the control condition, I_h was recorded without adding any compound, followed with perfusion with a 5 mL $3 \mu\text{M}$ KT5720 solution into the recording chamber, which dramatically attenuated the whole-cell I_h . At the end of recording, $10 \mu\text{M}$ ZD7288 (a selective HCN channel inhibitor) was added into the bath solution, in the presence of ZD7288, the remained inward current was totally inhibited (Fig. 1A). To confirm that the effect of KT5720 on I_h being attributed to PKA inhibition, the same experiments were carried out with H89, it had the same effect as KT5720 (Fig. 1B), which confirmed KT5720 attenuates I_h via PKA inhibition. Furthermore, to test whether KT5720 blocks I_h

through HCN channel inhibition, ZD7288 was applied to DRG neurons in the absence of KT5720. The result was consistent with our hypothesis, ZD7288 blocked whole-cell I_h .

HCN channel activation curve was leftward shifted and its current density was significantly reduced by PKA inhibition

In the above paragraph, we already demonstrated that PKA inhibition with KT5720 induced a dramatic reduction of I_h . This inhibition not only influences the current amplitudes, but also has an impact on the channel active threshold. The aftermath is the HCN channel activation curve shifted to the negative direction. Those points were obtained from the tail current amplitudes following application of hyperpolarizing voltage steps, and were normalized to the largest one at -140 mV. The activation curves have been fitted with a Boltzmann function. As illustrated in Fig. 2B, the HCN channel $V_{1/2}$ changed from -81.91 mV (control) to -100.85 mV (KT5720) ($n=9$, $N=6$). Furthermore, in the presence of $3 \mu\text{M}$ KT5720, the I_h density was declined as well. To ensure that the HCN channel has been fully activated, we chose -140 mV as the statistical analysis voltage, the current amplitudes averaged from the last 100 ms of the step-stimulation. Statistic results showed that the I_h density decreased from 16.82 ± 1.77 (control) to 3.57 ± 0.52 (KT5720) ($n=6$, $N=5$; $***p < 0.005$) (Fig. 2C).

KT5720 ($3 \mu\text{M}$) slows down HCN channel activation kinetics

Since KT5720 ($3 \mu\text{M}$) attenuated I_h amplitude and increased its activation threshold, thus shifting the $V_{1/2}$ to more negative potentials. We further postulated that PKA inhibition with KT5720 can slow down I_h activation kinetics. I_h was

FIG. 1. Whole-cell recording of I_h on freshly isolated DRG neurons. (A) I_h recorded under control condition (up); After $3 \mu\text{M}$ KT5720 was added into the bath solution, I_h was robustly attenuated (middle); the residual I_h was completely blocked after $10 \mu\text{M}$ ZD7288 was added into the bath solution (bottom). (B) Another PKA inhibitor H89 has similar effect on I_h as KT5720. (C) ZD7288 blocked I_h without PKA inhibition. DRG, dorsal root ganglion; PKA, protein kinase A.

FIG. 2. KT5720 significantly shifted HCN channel activation curve to the hyperpolarization direction and decreased its current density. **(A)** Original recording, the enlarged tail currents, and the corresponding protocol. **(B)** Activation curves in the presence (●) and absence (○) of KT5720. The activation curve was obtained from the tail current amplitude, the conductance (G) at each step voltage was standardized to the conductance at -140 mV ($G_{\max}$). Under control condition, $V_{1/2}$ was -81.91 mV, after $3 \mu\text{M}$ KT5720 was added to the bath solution, it shifted to -100.85 mV ($n=9$, $N=6$). **(C)** Summary result of KT5720 reduced the current density. Current density was obtained at -140 mV, the last 100 ms of the step recording was averaged as the current amplitude. Statistic result showed the current density was reduced from 16.82 ± 1.77 (control) to 3.57 ± 0.52 (KT5720) ($n=6$, $N=5$; $***p < 0.005$). HCN, hyperpolarization-activated cyclic nucleotide gated.

elicited with a sustained -130 mV stimulation for 2 s. To explore the effect of KT5720 on I_h activation, we first compared the original recording between control and KT5720 ($3 \mu\text{M}$). The activation time constant was fitted with a mono-exponential equation ($I = I_o + Ae^{-(x-x_o)/\tau}$), within which, τ is the time constant, x represents the time in ms, and I is the current. Our result demonstrated that I_h activation time constant was much longer in the presence of $3 \mu\text{M}$ KT5720 (Fig. 3A). Moreover, statistic results showed that the Tau value changed from 464.5 ± 35.86 ms (control) to 4164.5 ± 304.33 ms (KT5720) ($n=11$, $N=7$; $***p < 0.005$) (Fig. 3B).

PKA inhibition with KT5720 ($3 \mu\text{M}$) reduced DRG neurons excitability

At the end of AP, it was believed that I_h can depolarize the membrane potential to the threshold thus inducing a new AP (Accili *et al.*, 2002). It also reported that the HCN channel

selective inhibitor ZD7288 can attenuate DRG neuron excitability through inhibiting AP (Chaplan *et al.*, 2003). We further hypothesize that KT5720 inhibiting I_h may also have an impact on DRG neuron excitability, therefore, current-clamp recordings were implemented on six rats as shown in Figure 4A. APs were evoked by a 2 s depolarize current, which confirmed our hypothesis. As demonstrated in Figure 4A, after application with $3 \mu\text{M}$ KT5720, the APs were profoundly inhibited. Furthermore, AP recording was also carried out in the presence of $10 \mu\text{M}$ ZD7288. As illustrated in Figure 4B, KT5720 did not have a significant effect under this condition, which confirmed that KT5720 attenuates APs via HCN channel inhibition. AP threshold was changed from -25.25 ± 1.21 mV under control condition to -22.42 ± 1.54 mV in the presence of $3 \mu\text{M}$ KT5720 ($n=6$, $N=6$) (Fig. 4C). Moreover, AP overshoot amplitude significantly decreased from 23.77 ± 2.12 mV (control) to 6.93 ± 1.39 mV (KT5720) ($n=6$, $N=6$; $**p < 0.01$) (Fig. 4D).

FIG. 3. The activation time constant of HCN channel was dramatically increased by $3 \mu\text{M}$ KT5720. **(A)** Original recording showed I_h recorded from the same cell before and after perfusion with $3 \mu\text{M}$ KT5720, I_h was elicited from a holding potential of -40 mV, then hyperpolarized to -130 mV and sustained for 2 s. **(B)** Statistic result demonstrated that I_h time constant (tau) was 464.5 ± 35.86 ms under control condition. After perfusion with $3 \mu\text{M}$ KT5720, it increased to 4164.5 ± 304.33 ms ($n=11$, $N=7$; $***p < 0.005$), data are presented as mean $\pm$ SEM.

FIG. 4. The effect of KT5720 on DRG neuron action potential (AP). **(A)** AP was recorded under the condition without (up) or with (down) $3\ \mu\text{M}$ KT5720. A 2 s depolarization current was injected on DRG neurons to elicit AP through the recording electrode. **(B)** In the presence of $10\ \mu\text{M}$ ZD7288, APs were recorded without (up) or with (down) $3\ \mu\text{M}$ KT5720. **(C)** Statistic result showed that AP threshold changed from $-25.25 \pm 1.21\ \text{mV}$ (control) to $-22.42 \pm 1.54\ \text{mV}$ (KT5720) ($n=6$, $N=6$; $*p < 0.05$). **(D)** Histograms demonstrated that DRG neuron AP overshoot amplitude was $23.77 \pm 2.12\ \text{mV}$ (before) and $6.93 \pm 1.39\ \text{mV}$ (after) perfusion with $3\ \mu\text{M}$ KT5720, AP overshoot was taken from above $0\ \text{mV}$ ($n=6$, $N=6$; $**p < 0.01$).

The effect of KT5720 on HCN single-channel properties

To the best of our knowledge, no published literatures reported single-channel recording on freshly isolated DRG neurons so far. Therefore, to further reveal the mechanism of the PKA pathway on regulating HCN channel activation, cell-attached single-channel recording experiments were implemented as illustrated in Figure 5A. To eliminate irrelevant impacts, only those original gigaseal $\geq 10\ \text{G}\Omega$ were considered for further recording. After gigaseal formation, 15 more minutes were waited to ensure the seal was stable. Moreover, no rundown was observed under control condition, with $10\ \mu\text{M}$ ZD7288 added to ensure the recorded currents were I_h (data did not show). The open probability has a voltage-dependent characteristic, which showed it increased from 0.21 at $-50\ \text{mV}$ to 0.91 at $-140\ \text{mV}$, and the $V_{1/2}$ was $-82.18\ \text{mV}$ (Fig. 5), which was consistent with our whole-cell recording result and

the published report as well (Simeone *et al.*, 2005). In the presence of $3\ \mu\text{M}$ KT5720, the voltage-dependent characteristic of P_o was totally dampened. At $-90\ \text{mV}$, KT5720 inhibited P_o for 71%, it also slightly attenuated the current amplitude from $1.06 \pm 0.18\ \text{pA}$ (control) to $1.02 \pm 0.13\ \text{pA}$ (KT5720). Moreover, before and after application of $3\ \mu\text{M}$ KT5720, HCN channel conductance decreased from $12.41 \pm 0.69\ \text{pS}$ to $11.54 \pm 0.76\ \text{pS}$, but except for P_o neither the changes of current amplitude nor channel conductance were statistical significant ($n=13$, $N=9$) (Table 1). So, our data showed that PKA inhibition with KT5720 reduced HCN channel P_o .

PKA inhibition with KT5720 ($3\ \mu\text{M}$) reduced intracellular Ca^{2+} concentration

Previous study showed that the HCN channel activity was relevant to intracellular Ca^{2+} (Hagiwara and Irisawa, 1989),

FIG. 5. PKA inhibition induced reduction of HCN single-channel open probability. **(A)** HCN single-channel currents before (up) and after (down) perfusion with $3\ \mu\text{M}$ KT5720. Single-channel I_h was elicited with a $-90\ \text{mV}$ hyperpolarize stimulation. I_h was recorded with a cell-attached mode. The data sampling rate was 10 KHz and filtered with $-3\ \text{dB}$ 4-hole Bessel filter at 2 KHz. This original recording showed $3\ \mu\text{M}$ KT5720 tremendously inhibited HCN single-channel currents. **(B)** Analyses results showed KT5720 ($3\ \mu\text{M}$) enormously inhibited the voltage-dependent characteristic of P_o ($n=13$, $N=9$).

TABLE 1. SUMMARY EFFECTS OF KT5720 ON HCN SINGLE-CHANNEL PARAMETERS

	P_o	Current amplitude (pA)	Channel conductance (pS)
Control	0.85 ± 0.11	1.06 ± 0.18	12.41 ± 0.69
KT5720	$0.24 \pm 0.07^{**}$	1.02 ± 0.13	11.54 ± 0.76

$^{**}, p < 0.01$.

HCN, hyperpolarization-activated cyclic nucleotide gated.

which hints PKA-induced I_h inhibition may be attributed to the change of the intracellular Ca^{2+} level. To explore the effect of KT5720 on DRG neuron intracellular Ca^{2+} level, real-time calcium imaging experiments were implemented on freshly isolated DRG neurons. Fura 2-AM (a ratiometric and UV light-excitable dye) was used as the intracellular Ca^{2+} indicator, the dual-wave excitation UV lights were generated with a TILL's Polychrome V light source. Recording coverslips were coated with poly-L-lysine to fix the cells. At first, the experiment presented in Figure 6A was performed. In the control part, great care was taken to ensure that no intracellular degradation occurred. As showed in Figure 6A, application of KT5720 (3 μ M) profoundly reduced the intracellular Ca^{2+} level, similar results as illustrated in Figure 6A also were obtained from 12 other cells. The fluorescent ratio (340/380 nm) under control conditions was 0.80 ± 0.05 ($n = 13$, $N = 4$). Thereafter, the fluorescent ratio decreased to 0.64 ± 0.06 in the presence of 3 μ M KT5720 ($n = 13$, $N = 4$; Fig. 6B). Thus, application of KT5720 reduces the DRG neuron intracellular Ca^{2+} level, so, PKA inhibition induced I_h attenuation, at least partly, due to reduction of intracellular Ca^{2+} .

Discussion

In this study, we provided electrophysiological and calcium imaging data, which strongly suggest that PKA is a pivotal regulator of the HCN channel, thus controlling DRG neuron excitability. To the best of our knowledge, our study is the first to illuminate the physiological role and effect of PKA inhibition on the HCN channel activity on freshly isolated DRG neurons. Based on our data, we can draw these conclusions: (1) KT5720 inhibits HCN channel whole-cell

currents, I_h thereafter has a more hyperpolarized $V_{1/2}$, a decreased current density, and a slower activation time constant as well; (2) The PKA inhibitor KT5720 inhibits HCN single-channel currents, significantly reduced the P_o value, which is consistent with our whole-cell experiment result; (3) KT5720 attenuates I_h associated with the reduction of intracellular Ca^{2+} concentration, which further induced DRG neuron excitability reduction.

We observed robust attenuation of whole-cell HCN channel currents by the PKA inhibitor KT5720 (Fig. 1). When compared the parameters between control and in the presence of KT5720 conditions, the $V_{1/2}$ decreased 18 mV, which is in line with the published data, which addressed the effect of PKA on the HCN channel activity in mice sinoatrial nodes (Liao *et al.*, 2010). It is well known that cAMP is a critical and ubiquitous messenger that is involved in many intracellular regulation mechanisms (Kronstad *et al.*, 2011; Lania *et al.*, 2012; Billington *et al.*, 2013). An increased intracellular cAMP level was reported potentiated both transferred and native I_h (Gao *et al.*, 2012; Zong *et al.*, 2012). On the contrary, reduction of the cAMP concentration would induce I_h decline (Chen *et al.*, 2005). However, whether the cAMP/PKA pathway is involved in regulation of the HCN channel activity is still under controversy. For instance, Constantin's group reported that the Gonadotropin-Releasing Hormone-1 (GnRH-1) Neuronal activity was regulated by PKA, while independent of HCN channels. Specifically, the HCN channel protein was identified in GnRH-1 neurons, blockade of HCN channel with ZD7288 significantly attenuated the efficiency of forskolin to stimulate GnRH-1 neurons, whereas PKA inhibition did not have this effect. In other words, PKA inhibition has no effect on HCN channel activity (Constantin and Wray, 2008). On the contrary, another study showed that PKA inhibition with H89 profoundly blocked I_h in cultured rat olfactory receptor neurons (Vargas and Lucero, 2002). To clarify this issue, we carried out electrophysiological recording with the compound of KT5720 on freshly isolated rat DRG neurons.

Whole-cell patch-clamp data analyses demonstrated for the first time that KT5720 inhibiting I_h resulted in both the current density (Fig. 2) and the channel active kinetics reduction (Fig. 3). By analyzing the current density, we identified that it decreased 78.77% in the presence of KT5720. In

FIG. 6. KT5720 (3 μ M) induced DRG neuron intracellular Ca^{2+} concentration reduction. (A) Original recording illustrated that in the presence of 3 μ M KT5720, the intracellular Ca^{2+} concentration reduced. In this experiment, Fura-2 AM was chosen as dye, it stimulated with 340 and 380 nm ultraviolet lights, the emission light wave length was 510 nm. The Ca^{2+} concentration was represented with a ratio (340/380 nm) value. All the procedures were implemented in a light-resistant room. (B) Statistic results demonstrated that DRG neuron intracellular Ca^{2+} decreased from 0.80 ± 0.05 (control) to 0.64 ± 0.06 (KT5720) ($n = 13$, $N = 4$; $^{**}p < 0.01$).

addition, the HCN channel activation time constant increased 796.5% (Fig. 3). PKA-dependent inhibition of transferred TRPC5 channel was reported in HEK cells (Sung *et al.*, 2011), and previous study showed KT5720 inhibits rapid component $I(Kr)$ of cardiac delayed rectifier potassium currents in guinea pigs as well (Wang *et al.*, 2009). However, the effect of PKA inhibition on HCN channel activity remains to be fully explored. Here we revealed the key role of PKA regulating the HCN channel activity by conducting whole-cell patch-clamp experiments on freshly isolated DRG neurons.

DRG neurons excitability is essential to many important physiological procedures. For instance, it is well established that the pathophysiology of neuropathic pain is associated with altered DRG neuron excitability (Dib-Hajj *et al.*, 2008; Miller *et al.*, 2009); a rodent study indicated that DRG neuronal excitability also has impacts on visceral hypersensitivity (Xu *et al.*, 2012); as well as regulating DRG neuronal excitability can change the sensory innervation of the urinary bladder thus controlling the bladder's storage and void urine (Sun *et al.*, 2010). Furthermore, a number of ion channels have been reported to be involved in regulating DRG neuron excitability. Impaired $Na(v)1.7$ induced human DRG neuron hyperexcitability (Han *et al.*, 2012). A recent study in rat DRG neurons indicated that brain-derived neurotrophic factor levels increased under nerve injured conditions would suppress the BK channel activity, which further induced DRG neuron abnormal hyperexcitability (Cao *et al.*, 2012). Selective Kv subunit inhibition demonstrated to be able to enhance DRG neuron excitability too (Lee *et al.*, 2012). It was unanimously agreed that the HCN channel was extensively expressed in DRG neurons (Kouranova *et al.*, 2008), combined with its unique characteristics, which strongly hints that the HCN channel probably plays a key role in regulating DRG neuron function and more attention should be deserved to be paid on this subject. However, the function of the HCN channel and associated cAMP/PKA pathway in DRG neuron excitability remains to be elucidated. To date, only a handful of groups have carried out very preliminary studies on this topic (Wan, 2008; Wang *et al.*, 2012; Weng *et al.*, 2012). To address this critical issue, we conducted whole-cell current-clamp recording on freshly isolated DRG neurons. Our data confirmed our hypothesis that KT5720 not only robustly inhibited AP overshoot amplitude, but also increased its threshold (Fig. 4), which therefore indicated HCN channel and associated cAMP/PKA pathway were critical in regulating DRG neuron excitability.

To further investigate the effect of PKA inhibition with KT5720 in attenuating the HCN channel activity, cell-attached patch-clamp single-channel recording was implemented. Our result showed KT5720 significantly decreased the P_o value of the HCN channel (Fig. 5), which is compatible with our whole-cell recording results, and further confirmed our hypothesis that KT5720 selectively inhibits PKA would attenuate the HCN channel activity. Therefore, PKA is an important electrophysiological target to regulate the HCN channel activity. To date, HCN single-channel biophysical properties in native DRG neurons are still largely unknown. Those investigations conducted on recombination, cardiomyocyte and atrialmyocyte (Michels *et al.*, 2005, 2008a, 2008b) cannot be transferred to freshly isolated DRG neurons directly. Here we reported a HCN single-channel conductance of ~ 12 pS, which

is in line with the study conducted also with a cell-attached mode (Michels *et al.*, 2005). We chose the cell-attached mode, which can prevent dialysis and thereby maintain the intracellular circumstances; thus, our results have more electrophysiological significances.

The Ca^{2+} signal is the most important second messenger, which is relevant to both normal and pathophysiological processes (Leybaert and Sanderson, 2012; Skupin and Thurley, 2012). Recent study revealed PKA inhibition blocked T-type Ca^{2+} currents and reduced membrane excitability in mice DRG neurons (Wang *et al.*, 2011). However, the effect of PKA inhibition on freshly isolated rat DRG neuron cellular Ca^{2+} remains to be determined. Our result showed KT5720 (3 μ M) reduced intracellular Ca^{2+} levels on freshly isolated DRG neurons, which is in agreement with studies on neonatal rats (Hou and Wang, 2001). In addition, recent study revealed that Ca^{2+} -activated adenylyl cyclases is a new player on regulating HCN channel function (Kryukova *et al.*, 2012), and its combination with HCN2 could generate more efficient biological pacing (Boink *et al.*, 2012). Moreover, evidence indicated that HCN channel can directly conduct Ca^{2+} , although tiny, it contributes significantly the relevant biophysical functions (Michels *et al.*, 2008a). Taken together, even though our study cannot exclude the possibility that other ion channels and receptors may also be involved in this regulation, our effort provided the initial evidence that intracellular Ca^{2+} involved in the PKA inhibition induced I_h attenuation process.

In summary, this is the first study that addressed the effect of PKA inhibition on the HCN channel activity and further elucidated the effect on DRG neuron excitability. Our whole-cell and single-channel electrophysiological data indicated that PKA inhibition robustly attenuated the HCN channel activity. We provided further evidence that KT5720 selectively inhibits PKA reduced the intracellular Ca^{2+} level and further reduced DRG neuron excitability. Thus, our data strongly suggests PKA is a pivotal mediator of the HCN channel activity and DRG excitability. Therefore, it may represent a potential pharmacological target for the treatment of HCN channel and DRG neuron-relevant diseases.

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Disclosure Statement

There is no conflict of interest between the authors.

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