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Full-length article

Slack and Slick K_{Na} channels are required for the depolarizing afterpotential of acutely isolated, medium diameter rat dorsal root ganglion neurons[1]

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Key words

Slack; Slick; Na^+-activated K^+ channels; depolarizing afterpotential; dorsal root ganglion

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Abstract

Aim: Na^+-activated K^+ (K_{Na}) channels set and stabilize resting membrane potential in rat small dorsal root ganglion (DRG) neurons. However, whether K_{Na} channels play the same role in other size DRG neurons is still elusive. The aim of this study is to identify the existence and potential physiological functions of K_{Na} channels in medium diameter (25–35 μm) DRG neurons. **Methods:** Inside-out and whole-cell patch-clamp were used to study the electrophysiological characterizations of native K_{Na} channels. RT–PCR was used to identify the existence of Slack and Slick genes. **Results:** We report that K_{Na} channels are required for depolarizing afterpotential (DAP) in medium sized rat DRG neurons. In inside-out patches, K_{Na} channels represented 201 pS unitary chord conductance and were activated by cytoplasmic Na^+ [the half maximal effective concentration (EC_{50}): 35 mmol/L] in 160 mmol/L symmetrical K^+_o/K^+_i solution. Additionally, these K_{Na} channels also represented cytoplasmic Cl^--dependent activation. RT–PCR confirmed the existence of Slack and Slick genes in DRG neurons. Tetrodotoxin (TTX, 100 nmol/L) completely blocked the DRG inward Na^+ currents, and the following outward currents which were thought to be K_{Na} currents. The DAP was increased when extracellular Na^+ was replaced by Li^+. **Conclusion:** We conclude that Slack and Slick K_{Na} channels are required for DAP of medium diameter rat DRG neurons that regulate DRG action potential repolarization.

Introduction

Na^+-activated K^+ (K_{Na}) channels were originally identified in cardiomyocytes and may provide protection against ischemia[1]. K_{Na} channels are accompanied by an increase in intracellular Na^+ and may be involved in action potential shortening during ischemia[2]. They have been described to have many different functions in various neurons[3–7]. K_{Na} channels are activated at resting states in quail trigeminal ganglion neuron[5]. The accumulation of intracellular Na^+ during a train of action potentials may result in the activation of K_{Na} channels in the soma of rat motor neurons[7]. It has also been proposed that Na^+ influx through voltage-gated Na^+ channels during a single action potential produces a transient activation of K_{Na} channels, resulting in action potential repolarization[8,9].

The molecular identity of native K_{Na} channels is considered as Slo2, originally called Slack (also termed Slo2.2)[10]. The second K_{Na} channel gene is called Slick (also termed Slo2.1), which is homologous to Slack[3, 10]. The Slick K_{Na} channel is activated rapidly in response to depolarization and cytoplasmic Cl^-. There is an ATP-binding site in the N-terminal regions of Slick. Slack and Slick K_{Na} channels could be activated by both Na^+ and Cl^-. However, the Slick channel has low sensitivity to Na^+, but high sensitivity to elevating the internal Cl^- concentration[3].

In rat dorsal root ganglion (DRG) neurons, the largest cell bodies Aα- and Aβ-type DRG neurons usually transmit proprioceptive and tactile information, while smaller cell bodies Aδ- and C-type DRG neurons usually transmit pain and thermal information[11]. Scroggs and Fox reported that T-type Ca^{2+} currents were lower in small (20-27 μm) diameter DRG cell bodies (100 pA-1 nA) than observed in medium diameter (33-38 μm) DRG cell bodies (1-6 nA), and were not observed in large (45-51 μm) diameter DRG cell bodies[12]. Their results suggest the different distribution of Ca^{2+} channels in different size DRG neurons. Bischoff *et al* reported that K_{Na} channels set and stabilize the resting potential, but do not participate in single action potentials in rat small (20-25 μm) DRG neurons[4]. However, whether K_{Na} channels play the same role in medium diameter DRG neurons is still unknown.

In the present study, rat medium DRG neurons (25-35 μm), which were acutely isolated from lumbar segments of vertebrate column (L4-6) were used to study the potential functions of K_{Na} channels. K_{Na} channels were detected in ~80% membrane patches which displayed classical characterizations like large single channel conductance, subconductance states, and a block of single channel currents at positive potentials. K_{Na} channels expressed distinct cytoplasmic Na^+ and Cl^- concentration-dependent activation in these classes of neurons. Moreover, we demonstrated the existence of Slack and Slick in DRG neurons by RT–PCR. Using tetrodotoxin (TTX, 100 nmol/L) to block Na^+ influx, we found K_{Na} currents were outward K^+ currents which might contribute to repolarization of DRG action potential. With regards to replacement extracellular Na^+ with Li^+, we conclude that the outward K_{Na} currents contribute to the depolarizing afterpotential (DAP) of rat medium diameter DRG neurons.

Materials and methods

DRG neuron isolation Three-to-five-week-old Wistar rats (male) were killed by decapitation. The lumbar segments of the vertebrate column were dissected and the lumbar L4, L5, and L6 DRG, together with the dorsal, ventral roots, and attached spinal nerves were taken out from the outside of the spinal column. These 6 DRG were transferred into iced Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island. NY, USA) 13.5 g/L, NaCl 2.15 g/L, HEPES 2.0 g/L pH 7.4, 320 mOsm) immediately. After the removal of attached nerves and surrounding connective tissues, DRG were minced with iridectomy scissors and incubated with enzymes, including 1 mL collagenase (type I; Sigma-Aldrich, St. Louis, MO, USA) 2 mg/mL, 1 mL trypsin (Type IX, Sigma) 0.5 mg/mL, and 50 μL DNase, 4 mg/mL [in calcium-free buffer with 4 mg/mL BSA(bovine serum albumin)] in a 37 ℃ shaking bath (170 r/min) for 35-40 min with gently mechanical trituration every 10 min. The addition of 8 mL of pre-incubated DMEM [including 20% FBS (fetal bovine serum)] was used to stop the enzymatic digestion. The isolated neurons were plated on 0.5 mg/mL poly-lysine coated glass coverslips and maintained in a 37 ℃ humidified incubator with 5% CO_2 for at least 2 h before use. The medium neurons with a diameter of 25-35 μm were used in the experiments.

Electrophysiology For whole-cell clamp, the pipette solution contained the following (in mmol/L): 140 K-gluconate, 20 KCl, 10 HEPES, 5 EGTA, 2 MgATP, and 0.3 Na_2GTP (pH 7.2 with KOH, and 300 mOsm). The external solution contained (in mmol/L): 145 NaCl, 2.5 KCl, 4 $MgCl_2$, 1 EGTA, 10 HEPES, and 10 glucose (pH 7.3 with NaOH, and 310 mOsm). To make the Na^+-free saline, 145 mmol/L NaCl was replaced with 145 mmol/L LiCl. For the inside-out recordings, the pipette extracellular solution contained (in mmol/L): 140 methanesulfonic acid, 150 KOH, 10 KCl, 10 HEPES, and 2 $MgCl_2$ (pH 7.2 with methanesulfonic acid). Testing solutions bathing the cytoplasmic face of the patch membrane contained (in mmol/L): 100 KCl, 60 KOH, 60 methanesulfonic acid, 5 EGTA, and 10 HEPES; 0, 20, 40, and 80 mmol/L NaOH was added for different concentrations of the Na^+_i solution (pH 7.2 with methanesulfonic acid). For different concentrations of Cl^-_i, redundant Cl^- was replaced with same quantity of methanesulfonic acid to make 10, 100, and 160 mmol/L Cl^-. Osmolarity was measured by a vapor pressure osmometer (Wescor INC., Logan, Utah, USA) and adjusted to 300–310 mOsm (pipette solution) and 310–330 mOsm (extracellular solution). All experiments were performed at room temperature (22–25 ℃)[13].

RT–PCR The RNeasy Mini Kit (QIAGEN, Valentia, CA, USA) was used to extract the total RNA from the rat DRG[14]. The cDNA of the Slick and Slack were amplified by RT–PCR with the Qiagen OneStep RT–PCR kit (QIAGEN, Valentia, CA, USA). Two primers for amplifying Slack and Slick (the upstream primer 5´-CATAACTGCTATGAGGATGC-3´ and the downstream primer 5´-GTCTTGGCATCTGCCATGTAGTC-3´) were used in the RT–PCR reaction. The RT–PCR products were extracted by QIAquick Gel Extraction Kit (QIAGEN, Valentia, CA, USA) and then ligated into a pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA) for the sequence analysis.

Data analysis Data were analyzed with Igor 5.03 (Wavemetries, Lake Oswego, OR, USA), Clampfit (Axon Instruments Inc., Foster City, CA, USA), and SigmaPlot (SPSS, Chicago, IL, USA). Unless stated otherwise, the data are presented as mean±SEM. Significance was tested by Student's *t*-test, and differences in the mean values were considered significant at a probability of $P<0.05$.

The dose–response curve for the open probability (P_o) of K_{Na} was drawn according to the Hill equation $P_o=P_{(max)}/(1+[EC_{50}/[Na^+]_i]^n)$, where $P_{(max)}$ is the maximum P_o of the K_{Na} currents, and $[Na^+]_i$ is the concentration of cytoplasmic Na^+. EC_{50} and n denote the Na^+ concentration of the half-maximal effect and the Hill coefficient, respectively.

Results

K_{Na} channels in DRG neurons In our experiments, K_{Na} channels were present in approximately 80% in all inside-out patches. The representative single-channel currents were evoked by 80 mmol/L cytoplasmic Na^+ (Na^+_i) at different holding potentials which contained 2 opened K_{Na} channels (Figure 1A). The unitary chord conductance of the K_{Na} channel was 201±3.8 pS (n=8) by fitting the current–voltage relationship curve through the line function (Figure 1B). Under this condition, K_{Na} channels displayed linear open characterization from –100 to 0 mV. When the membrane potential was more positive than the potassium equilibrium potential, the single-channel currents exhibited inward rectification and opened in bursts (Figure 1A; +60 mV), during which they fluctuated between the fully open, closed, and the substates. This was proposed as the result of the Na^+ block of outward K_{Na} currents at positive potentials.

K_{Na} channels exhibited different Na^+ concentration-dependent activation in various neurons. The effect of different Na^+_i concentrations on the P_o of K_{Na} channels was studied in medium diameter DRG neuron cell bodies. Single-channel currents were not activated in the absence of Na^+_i, but could be gradually evoked in 20, 40, and 80 mmol/L Na^+_i (Figure 1C), although the activity of K_{Na} channels was not completely open in 80 mmol/L Na^+_i (n=12). The best fit of the data using the Hill equation obtained the following parameters: P_{max} (maximum P_o) is 0.62, EC_{50} is 35 mmol/L, and n (Hill coefficient) is 2.4 (Figure 1D). One possible explanation for the steep relationship between P_o and the Na^+_i concentration is that the binding of 2–3 Na^+ was necessary to open a K_{Na} channel. These results suggested that Slack K_{Na} channels exist in medium diameter DRG neurons.

Slack and Slick K_{Na} channels are reported to have overlapping distribution in the central neural system[6,15]. In order to understand the existence of Cl^--activated Slick channel in medium diameter rat DRG neurons, different concentrations of cytoplasmic Cl^- (10, 100, and 160 mmol/L) were used to study the single-channel currents in an inside-out clamp (Figure 1E). The single channel conductance of Cl^--activated K^+ channels is 182±1.2 pS at –100 mV with fre-

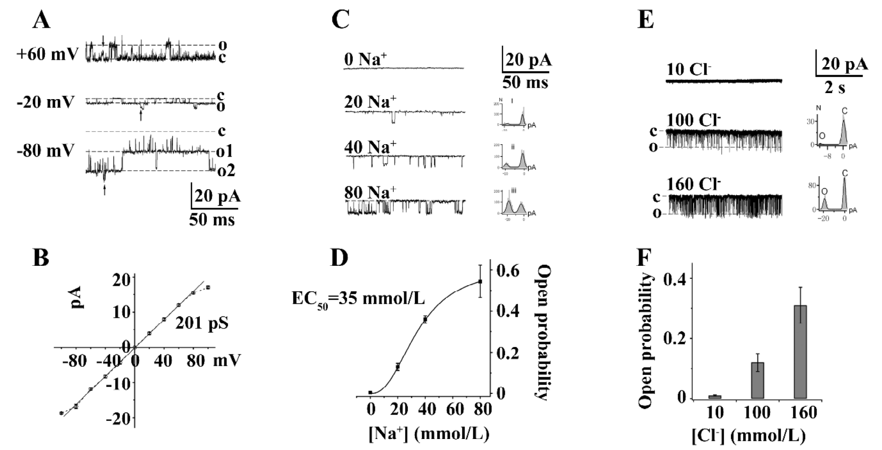

Figure 1. Na^+ activated K^+ conductance in DRG neurons. (A) single-channel currents were recorded from an inside-out patch activated by internal 80 mmol/L $[Na^+]_i$ and 100 mmol/L $[Cl^-]_i$ in 160 mmol/L symmetrical K^+_o/K^+_i solution at different holding potentials. C and O indicate the close and open states of the channel, and arrows indicate the subconductance states. (B) current–voltage relationship of single channel from multiple patches. Slope conductance is 201±3.8 pS (n=8) fitted by the line function. (C) currents activated by 0, 20, 40, and 80 mmol/L $[Na^+]_i$ at the cytoplasmic face of the patch (with 100 mmol/L $[Cl^-]_i$). Membrane potential was stepped to –100 mV from a holding potential of 0 mV. Point histograms taken from the current traces are shown on the right. Bin width is 0.25 pA, and the data were fitted by the sum of 2 Gaussian curves (i: 20 mmol/L $[Na^+]_i$, ii: 40 mmol/L $[Na^+]_i$, iii: 80 mmol/L $[Na^+]_i$). (D) P_o as a function of $[Na^+]_i$. P_o was calculated form 100–200 traces of different $[Na^+]_i$. Data were expressed as the mean±SEM (n=12) and fitted with the equation: $P_o=P_{max}/(1+[EC_{50}/[Na^+]_i]^n)$, where P_{max} (maximum P_o) is 0.62, EC_{50} is 35 mmol/L, and n (Hill coefficient) is 2.4. (E) currents activated by 10, 100, and 160 mmol/L $[Cl^-]_i$ at the cytoplasmic face of the patch (with 20 mmol/L $[Na^+]_i$). (F) open probability (P_o) as a function of $[Cl^-]_i$ was calculated form more than 200 traces of different $[Cl^-]_i$ (n=6).

quent subconductance currents. These Cl^--activated K^+ channels exhibited a similar burst, open mode like cloned Slick channels in Chinese Hamster Ovary cells[3]. The P_o of the Cl^--activated K^+ channels at different cytoplasmic Cl^- concentrations is shown in Figure 1F (n=6). The results suggested that Slick K_{Na} channels may also exist in medium diameter DRG neuron cell bodies.

Both slack and slick exist in DRG neurons by RT–PCR Total RNA from the rat DRG was extracted and then used as the RT–PCR template. The RT–PCR products amplified by primers were 2 bands according to the DNA electrophoresis. One of the 2 bands was approximately 250 bp, and another was approximately 350 bp. The RT–PCR products were subcloned into a pCR2.1-TOPO vector for DNA sequencing (Figure 2A). The resulting sequences showed a very high homology to the rat potassium channel subunit (Slack; PubMed NM_021853; Figure 2B) and rat Na^+- and Cl^--activated, ATP-sensitive potassium channel (Slick; PubMed NM_198762; Figure 2C). The results demonstrated that both Slack and Slick exist in rat DRG neurons.

Na^+ influx-dependent outward K_{Na} currents It has been reported that Na^+ influx through voltage-gated Na^+ channels during a single action potential could produce a transient activation of K_{Na} channels[8,9]. In order to study the physiological roles of K_{Na} channels in medium diameter DRG neurons, we used a single action potential recorded from medium diameter DRG neurons in a current clamp to simulate the physiological response of K_{Na} channels. In a free Ca^{2+} extracellular solution, the inward Na^+ current (I_{Na}) and the following outward K_{Na} current ($I_{K(Na)}$) were evoked (Figure 3A). In order to divide the I_{Na} from other potential inward currents, we used 100 nmol/L TTX to block the inward I_{Na} (Figure 3A). TTX completely blocked the inward I_{Na} (Figure 3A; n=6). The single action potential peak amplitude was 30 mV (Figure 3A), which is a lot lower than the TTX-sensitive Na^+ channels reverse potential (>50 mV). The following outward current was not I_{Na}, but a Na^+ influx-activated outward current, which was believed to be the Na^+-activated K^+ current[16,17]. Rat DRG neurons usually have continuous action potentials firing. We created a train of action potentials as a continuous stimulation waveform using HEKA Pulse and Igor software in which each was the same as the single action potential in Figure 3A (Figure 3B). Similar results were recorded in medium diameter DRG neurons (Figure 3B; n=6). K_{Na} channels currents were outward K^+ currents under physiological action potential firing, which

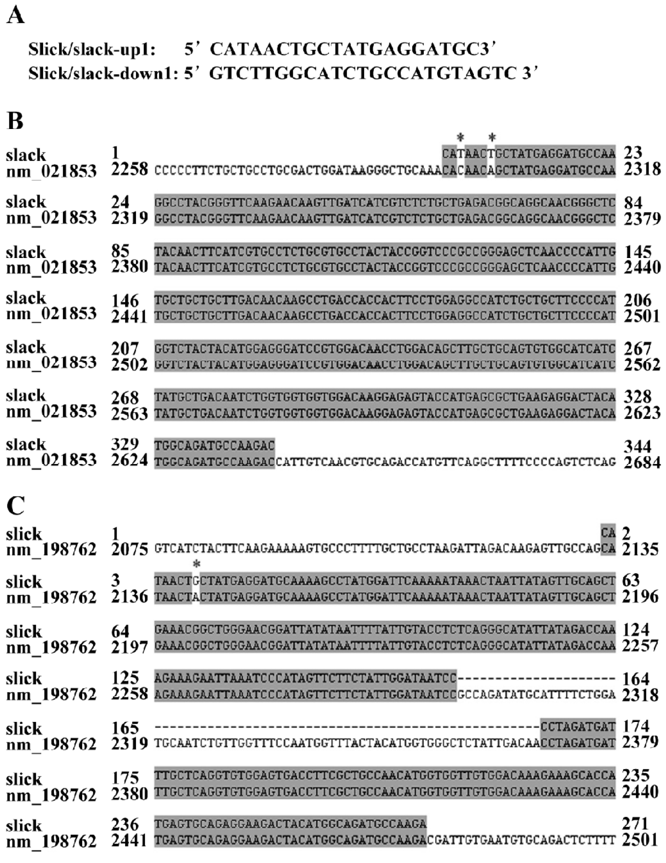

Figure 2. Both Slack and Slick exist in rat DRG neurons. (A) up and down primers sequences we used for the RT–PCR for both Slack and Slick. (B) sequences of Slack RT–PCR products in rat DRG compared with the published Slack sequence (PubMed NM_021853). (C) sequences of Slick RT–PCR products in rat DRG compared with the published Slack sequence (PubMed NM_198762).

suggested that they play an important role in medium diameter DRG action potentials transferring.

K_{Na} channels contribute to DAP in medium diameter DRG neurons The replacement of extracellular Na^+ with Li^+ is usually performed to study the assumed contribution of K_{Na} channels in the regulation of action potential in current clamps. Single action potentials were evoked by short (30 ms) current pulses of 0.5 nA. The shape of action potential (AP) waveforms was compared from a certain cell in 145 mmol/L Na^+ or replacement by 145 mmol/L Li^+ and washout with 145 mmol/L Na^+ at intervals of 1–2 min, respectively (Figure 4A). The absolute value of DAP was increased from 17.5±0.8 mV to 27.1±1.2 mV after the replacement of extracellular Na^+ with Li^+, but action potential duration (APD) at 0 mV ($APD_{0\,mV}$) and after hyperpolarizing potential (AHP) were not affected (Figure 4B). Washing out with 145 mmol/L Na^+ could recover the DAP of action potentials. The results indicated that K_{Na} channels are required for action potential repolarization in medium diameter rat DRG neurons.

Discussion

DRG neurons transfer much complex sensory informa-

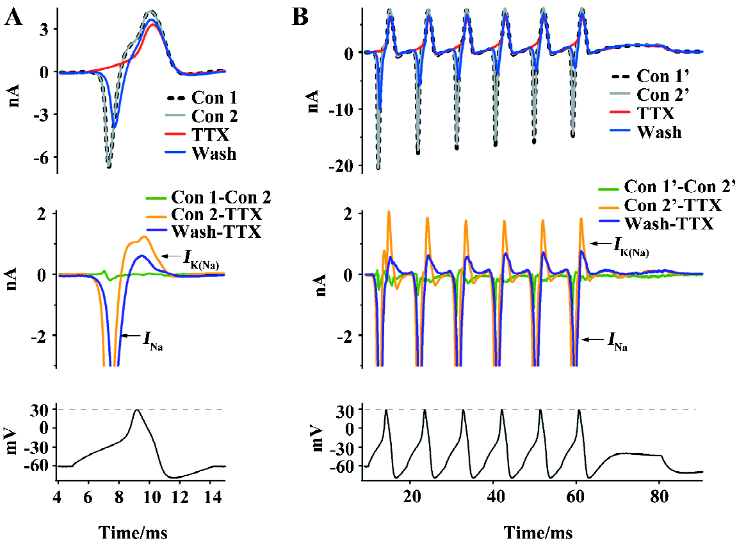

Figure 3. TTX blocked K_{Na} currents evoked by action potentials. (A) representative whole-cell currents evoked by a single action potential. Con 1 and Con 2 were 2 control currents recorded at intervals of 1 min which had no difference. TTX completely blocked the inward Na^+ current and the following outward K_{Na} current. TTX-sensitive inward Na^+ current and following Na^+-dependent outward K_{Na} current were obtained by Con 2–TTX (n=6). Na^+ currents and following outward K_{Na} current were restored partially after washing out TTX (wash–TTX, n=6). A single action potential stimulation waveform is shown at the bottom. (B) a train of action potentials (6 action potentials) as a stimulation waveform was used to simulate and evoke the rough physiological inward Na^+ currents. Con 1´ and Con 2´ were 2 control currents recorded at intervals of 1 min which had no difference. Continuous Na^+ and K_{Na} currents were the same as in (A) (Con 2´–TTX, n=6). A stimulation waveform of 6 action potentials is shown at the bottom. Data were recorded at intervals of 1 min.

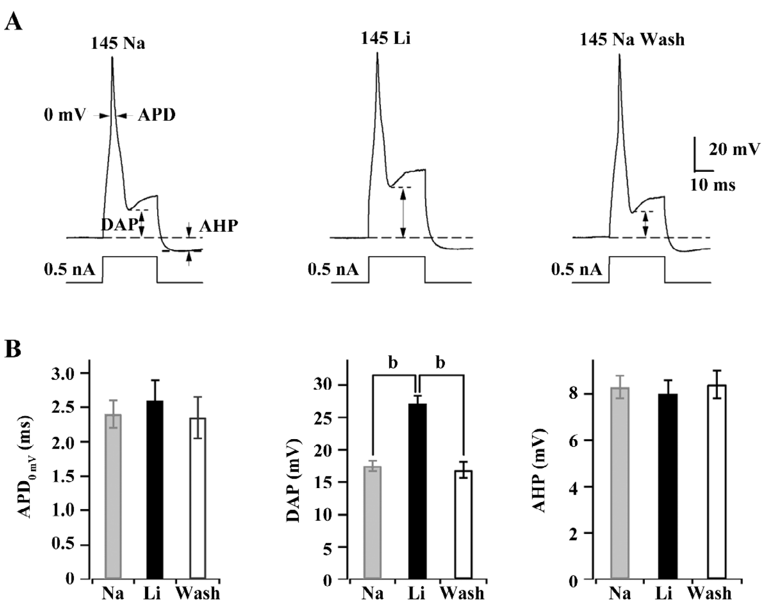

Figure 4. K_{Na} channels were required for DRG DPA. (A) representative single action potentials evoked by a 0.5 nA current pulse for 30 ms were recorded in 145 mmol/L Na^+, and placement by 145 mmol/L Li^+ and 145 mmol/L Na^+ washout solutions in free Ca^{2+} extracellular solution, respectively. (B) characteristic parameters of a single action potential were compared in Na^+, Li^+, and washout extracellular solution: $APD_{0\,mV}$, DAP absolute value, AHP absolute value. DAP increased after extracellular Na^+ was replaced with Li^+, but $APD_{0\,mV}$ and AHP did not change (n=8). ^bP<0.05.

tion like pain, temperature, proprioceptive, and tactile information[8,9]. Different sensory information might depend on different size DRG neurons. Sensory information transfer was thought to be coded on the DRG action potential firing frequency, amplitude, and firing pattern. The shape of neuron action potentials was dependent on the opening of various ion channels on the cell periphery. In this study, the results showed that the Slack and Slick Na^+-activated K^+ channels existed and contributed to action potential DAP in acutely isolated, medium diameter rat DRG neuron cell bodies. Native K_{Na} channels had distinct properties similar with the cloned Slack and Slick channels, including a large single channel conductance (201 pS; Figure 1), sensitivity to intracellular Na^+ and Cl^- (Figure 1), multiple subconductance states, and a block of single channel currents at high positive potentials (Figure 1)[3,10]. RT–PCR demonstrated that both Slack and Slick K_{Na} channels existed in the DRG neurons (Figure 2). This method has been successfully used to demonstrate that the β2-subunit, but not β3-subunit, induces the inactivation of calcium-activated potassium (BK) channel in small DRG neurons[14]. However, how K_{Na} channels are opened under physiological conditions and whether K_{Na} channels contribute to regulate the physiological functions in medium diameter rat DRG neurons is unknown. Using a real action potential stimulation waveform, whole-cell K_{Na} currents appeared to be activated by Na^+ influx through TTX-sensitive Na^+ channels (Figure 3). The transient currents did not appear to be the results of the lack of space clamp because the neurons examined were small with very short processes and their series resistance was compensated. Additionally, Na^+ current was blocked reversibly by TTX. In a current clamp, we demonstrated that the K_{Na} channels were required for action potential DAP in medium diameter rat DRG neurons by replacing Na^+ with Li^+ (Figure 4). The effect of replacing Na^+ on the DAP did not appear to be a result of an action of the Na^+–Ca^{2+} exchanger or the Na^+–H^+ exchanger because of the intracellular solution containing EGTA or a high HEPES concentration.

Functions of K_{Na} channels Over the past several years, many physiological functions of K_{Na} channels have been proposed. One of the surprises is that such channels can act over a wide range of time scales to influence the action potential firing pattern of neurons. The physiological roles of K_{Na} channels have been difficult to characterize because of the lack of specific K_{Na} channel blockers. However, there have been several studies showing that K_{Na} channels contribute to the regulatory neuronal activity and the action potential waveform to produce adaptation of firing rates and to set the resting membrane potential[5,16]. The kinetic properties of Slack channels suggest that they contribute to currents that develop slowly during maintained neuronal firing. Na^+-dependent slow AHP lasting many seconds have been described in various neurons depending on Na^+ influx and following repetitive neuronal firing[18,19]. K_{Na} channels participate in the DAP following a single action potential in rat hippocampal CA1 pyramidal cells[20]. The size of the DAP was controlled by the activation of an opposing K_{Na} conductance that was detected as early as 5–10 ms after a single action potential[20]. In this work, we found that the K_{Na} channels were activated by a Na^+ influx evoked by a single action potential in normal Ca^{2+} free extracellular saline in medium diameter DRG neurons. The activated K_{Na} currents were outward following Na^+ influxes. Using ionic replacement, we demonstrated that K_{Na} channels were required for DAP, but not $APD_{0\,mV}$ or AHP.

A recent study that found that the activity of Slack channels can be enhanced by estradiol raises the possibility that the activation of K_{Na} channels contributes to estradiol-dependent neuroprotection in ischemia[21]. Although evidence of the possible role of K_{Na} in pathologies is circumstantial, it raises that possibility that these channels could be therapeutically useful drug targets. Similarly, the existing function of Slack and Slick K_{Na} channels in medium diameter rat DRG neurons may be useful in the research of therapeutic drugs for the treatment of pain.

Author contributions

Shang-bang GAO and Jiu-ping DING designed research; Shang-bang GAO and Ying WU performed research; Cai-xia LÜ and Zhao-hua GUO contributed new analytical reagents and tools; Shang-bang GAO and Chen-hong LI analyzed data; Shang-bang GAO and Jiu-ping DING wrote the paper.

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