Journal of Cellular Physiology

Characterization of Voltage- and Ca^{2+}-Activated K^+ Channels in Rat Dorsal Root Ganglion Neurons

WEI LI,[1] SHANG-BANG GAO,[1] CAI-XIA LV,[1] YING WU,[1] ZHAO-HUA GUO,[1] JIU-PING DING,[1]* AND TAO XU[1,2]**

[1] *1Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei, 430074, China*

[2] *National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*

Auxiliary β-subunits associated with pore-forming Slo1 α-subunits play an essential role in regulating functional properties of large-conductance, voltage- and Ca^{2+}-activated K^+ channels commonly termed BK channels. Even though both noninactivating and inactivating BK channels are thought to be regulated by β-subunits (β1, β2, β3, or β4), the molecular determinants underlying inactivating BK channels in native cells have not been extensively demonstrated. In this study, rβ2 (but not rβ3-subunit) was identified as a molecular component in rat lumbar L4-6 dorsal root ganglia (DRG) by RT-PCR responsible for inactivating large-conductance Ca^{2+}-dependent K^+ currents (BK_i currents) in small sensory neurons. The properties of native BK_i currents obtained from both whole-cell and inside-out patches are very similar to inactivating BK channels produced by co-expressing mSlo1 α- and hβ2-subunits in Xenopus oocytes. Intracellular application of 0.5 mg/ml trypsin removes inactivation of BK_i channels, and the specific blockers of BK channels, charybdotoxin (ChTX) and iberiotoxin (IbTX), inhibit these BK_i currents. Single BK_i channel currents derived from inside-out patches revealed that one BK_i channel contained three rβ2-subunits (on average), with a single-channel conductance about 217 pS under 160 K^+ symmetrical recording conditions. Blockade of BK_i channels by 100 nM IbTX augmented firing frequency, broadened action potential waveform and reduced after-hyperpolarization. We propose that the BK_i channels in small diameter DRG sensory neurons might play an important role in regulating nociceptive input to the central nervous system (CNS).

J. Cell. Physiol. 212: 348–357, 2007.

Large-conductance, voltage- and Ca^{2+}-activated K^+ (BK) channels couple intracellular Ca^{2+} with cellular excitability. The BK channel is composed of four pore-forming α-subunits and is often associated with up to four β-auxiliary subunits (Ding et al., 1998). The association of tissue-specific β-subunits accounts for many phenotypic BK channels observed in native tissues, because β-subunits increase the apparent Ca^{2+} and voltage sensitivities of α-subunits, modify channel kinetics and alter their pharmacological properties (McManus et al., 1995; Wallner et al., 1995; Dworetzky et al., 1996; Tseng-Crank et al., 1996; Nimigean and Magleby, 1999; Wallner et al., 1999; Xia et al., 1999, 2000; Brenner et al., 2000; Meera et al., 2000a). Four members of β family have been identified by means of human EST databases, and two of them, β2 and β3, exhibit inactivation (Wallner et al., 1999; Xia et al., 1999, 2000). The physiological role of β subunits can be inferred from their impact on BK channels in native cells. The β1-, β2-, and β4-subunits have already been found and confirmed in many rat tissues such as smooth muscle, adrenal medulla, pancreas, and brain by comparing native BK currents to expressed ones (Xia et al., 1999, 2000; Uebele et al., 2000). Many studies have strongly demonstrated the existence of the β2-subunit in both the chromaffin and the pancreatic β cells (Li et al., 1999; Xia et al., 1999). The correspondence of functional properties between BK_i and Slo1 α/hβ2 currents has suggested that the β2-subunit is a key molecular component required for the inactivation behavior of the native BK_i channel, although other β-subunits may also exist in chromaffin cells (Xia et al., 2000). However, the native BK_i currents do exhibit some differences compared with those of expressed Slo1/β2-subunits (Wallner et al., 1999; Xia et al., 1999). For example, the half-activation voltage ($V_{0.5}$) of native BK_i currents in chromaffin cells indicates a somewhat rightward shift relative to those of expressed Slo1/β2 currents. These differences imply that BK_i channels in chromaffin cells may contain, on average, less than four β2-subunits per channel (Ding et al., 1998).

The small diameter dorsal root ganglia (DRG) neurons, which are likely to convey information of pain and temperature, not

Wei Li and Shang-Bang Gao contributed equally to this work.

Contract grant sponsor: National Science Foundation of China;
Contract grant numbers: 30470449, 30025023, 30270363, 30130230, 30470448.
Contract grant sponsor: Major State Basic Research Program of P.R. China;
Contract grant number: 2004CB720000.
Contract grant sponsor: CAS Project;
Contract grant number: KSCX2-SW-224.
Contract grant sponsor: Li Foundation and the Sinogerman Scientific Center.

*Correspondence to: Jiu-Ping Ding, School of Life Science and Technology, Huazhong University Science and Technology, Wuhan, 430074 Hubei, China. E-mail: jpding@mail.hust.edu.cn

**Correspondence to: Tao Xu, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China. E-mail: xutao@ibp.ac.cn

Received 26 July 2006; Accepted 4 December 2006

DOI: 10.1002/jcp.21007

only express distinct voltage-gated K^+ (K_V) channels, but also BK channels (Scholz et al., 1998; Rasband et al., 2001). However, the composition and functional role of BK channels in DRG neurons have not been extensively studied. To investigate the composition of BK channels in small DRG neurons, we firstly separated the BK_i currents from the total potassium currents by the blockers of BK channels, ChTX and IbTX, and then validated the rβ2-subunits by RT-PCR and trypsin. Based on the single-channel data, the properties of BK_i currents in DRG sensory neurons are very similar to those of mSlo1/hβ2 currents expressed in oocytes (Xia et al., 2003). Our results also suggested that the stoichiometry of the BK_i α- and auxiliary rβ2-subunits in DRG neurons was three rβ2-subunits per channel and that the population of BK_i channels in DRG cells comprise at least 57% of total apamin and 4-aminopyridine (4-AP) insensitive potassium current.

DRG neurons represent a diverse population with different sizes and characteristics designed to convey somatic and visceral sensory information from peripheral tissues to the spinal cord (Devor, 1999). Neurons with small- or medium-diameter axons and cell bodies are likely to convey information of pain and temperature, whereas neurons with large-diameter axons and cell bodies are more likely to convey mechanoreceptive information. Therefore, it is necessary to know whether BK_i channels really play a crucial role in the smaller-diameter DRG neurons. By means of current-clamp experiments with its specific blocker, IbTX, BK_i channels in the rat DRG neurons were found to regulate the firing frequency, waveform and after-hyperpolarization (AHP) of action potentials (APs). This is the first time that an inactivating voltage- and Ca^{2+}-acitvated K^+ (BK_i) channel regulated by auxiliary rβ2-subunits has been identified in sensory neurons. Consequently, it is necessary to consider and study the role of auxiliary rβ2-subunit in DRG neurons in the future.

Materials and Methods

Preparation of the rat small-diameter DRG cells

Three to five-weeks-old Wistar male rats were killed by decapitation. The lumbar segments of vertebrate column were dissected and the L4, L5, and L6 (L4-6) DRGs together with dorsal and ventral roots and attached spinal nerves were taken out from the spinal column. These ganglia were selected because sciatic DRG neurons are normally located in lumbar ganglia L3–6 and nearly 98–99% of sensory fibers in the sciatic nerve have cell bodies in the L4 and L5 DRGs (Swett et al., 1991). The six ganglia were immediately transferred into iced Dulbecco's Modified Eagle's Medium (DMEM, Gibco: DMEM 13.5 g/L, NaCl 2.15 g/L, HEPES 2.0 g/L) [pH = 7.4, 320 mOsmol/kg]. After the removal of attached nerves and surrounding connective tissues under a dissecting microscope, the ganglia were minced with iridectomy scissors and incubated with enzymes including 1 ml collagenase (Type I, 2 mg/ml), 1 ml trypsin (Type IX, 0.5 mg/ml), and 50 μl DNase (4 mg/ml) (in calcium-free KRBB buffer with 4 mg/ml BSA) at 37°C in a shaking bath (170 rpm) for 40 min. Gentle mechanical trituration by pipetting was performed every 10 min. The enzymatic digestion was stopped after 45 min by adding 8 ml preincubated DMEM medium (including 16% FBS). The isolated neurons were plated on glass coverslips coated with 0.5 mg/ml polylysine and maintained still at 37°C in a humidified incubator with 5% CO_2 at least for 2 h before use.

Isolation of the BK-type rβ2 cDNA by RT-PCR

RNeasy Mini Kit (QIAGEN) was used to extract total RNA from the rat DRG. The cDNA of the BK-type rβ2-subunits was amplified by RT-PCR with a Qiagen OneStep RT-PCR Kit (QIAGEN). Two primers for amplifying rβ2-subunits (the upstream primer 5′-ATGTTTATATGGACCAGTGGCCGGAC-3′ and the downstream primer 5′-TTATCTGTTGATTCGTTGGATCCTCTCACAAAG-3′) were used in the RT-PCR reaction. The RT-PCR product was extracted by a QIAquick Gel Extraction Kit (QIAGEN) and then ligated into pCR2.1-TOPO vector (Invitrogen) for sequence analysis. Three pairs of primers were designed to extract the BK-type rβ3 or the rβ2 splicing variant in the rat DRG. The pairs of primers used in the PCR reactions were 5′-ATGCAGCCCTTCAGCATCCCGGTCCAAA-TCACACTACAGG-3′ (upstream) with 5′-TCACAGCTCAGCCTGCTGGACTGCCACGTGTTGAATGTC-3′ (downstream) for isolating the full length of rβ3 (XM_227040), 5′-CTGGACTTTGCCTTCACCTGTG-3′ (upstream) with 5′-CACACTGAAAGGACAGATGTTGTGTTA-3′ (downstream) for probing a segment (451–847 bps) of the rβ3 cDNA, and the first-round primers 5′-ATTGAATTCGATCATCACTAGTCG-3′ (5′-outer) with 5′-CCACAGCTGAAGGAACAGTTAAATGTT-3′ (3′-outer) and the second-round primers 5′-GAACTTCATTGAATTCGATCATCA-3′ (5′-inner) with 5′-AATCTGCAGGGCACGCTGGGCTTCTTCTGTCC-3′ (3′-inner) for scanning a splicing variant of the rβ2 gene by 5′-RACE.

Patch CLAMP electrophysiology

The data were collected by an EPC-9 amplifier (HEKA Instruments, Lambrecht, Germany). In whole-cell experiments, uncompensated series resistance (Rs) was typically 2–4 MΩ, of which 90–95% was electronically compensated. In inside-out patches, the sampling frequency was 10 kHz and the filter frequency was 2–3 kHz. For whole-cell recording, the pipette solution contained the following (in mM): KCl 150, $MgCl_2$ 2.5, HEPES 10, EGTA 0.2, MgATP 2, Na_2GTP 0.3 (pH adjusted to 7.4 with KOH). The external solution contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose 10, (pH adjusted to 7.4 with NaOH). For making Ca^{2+}-free normal saline, 2 mM $CaCl_2$ was replaced with 2 mM $MgCl_2$ and supplemented with 0.1 mM EGTA. For inside-out recording, the pipette extracellular solution contained (in mM): 140 potassium methanesulfonate, 20 KOH, 10 HEPES, 2 $MgCl_2$ (pH adjusted to 7.0 with methanesulfonate). Test solutions bathing the cytoplasmic face of the patch membrane contained (in mM): 140 potassium methanesulfonate, 20 KOH, 10 HEPES (pH adjusted to 7.0 with methanesulfonate). EGTA (5 mM) was added for nominally Ca^{2+}-free solution. To make 10 μM free-Ca^{2+} solution, HEDTA (5 mM) and $CaCl_2$ (2.988 mM) were added. Appropriate free-Ca^{2+} concentration at a given buffer was calculated by the EGTAETC program (E. McCleskey, Vollum Institute, Portland, OR). Osmolarity was measured by vapor pressure osmometer (Wescor Osmometer, Logan, Utah) and adjusted to 290–300 mOsm/kg (internal solution) and 305–315 mOsm/kg (external solution). All experiments were performed at room temperature (20–25°C).

Reagents

Apamin (200 nM) and 4-AP (10 mM) were routinely added to extracellular solutions to minimize contamination by SK currents (Neely and Lingle, 1992) and voltage-dependent K^+ (K_V) currents, respectively (Goodman and Art, 1996). 4-AP, charybdotoxin (ChTX), and iberiotoxin (IbTX) were obtained from Sigma. To prevent nonspecific binding of the peptide toxins, 0.5% (w/v) bovine serum albumin (BSA) was added to test solutions containing apamin, ChTX, and IbTX, which were freshly diluted from a stock solution (50 μM). Unless otherwise stated, all reagents were purchased from Sigma.

Data analysis

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

Results

Properties of inactivating Ca^{2+}-dependent K^+ currents in DRG neurons

To record BK currents in DRG neurons, we adopted a double-pulse protocol to separate the total K^+ current into two components, BK + K_V and K_V, in whole-cell recordings as we did in rat chromaffin cells (Solaro et al., 1995). Obviously, the difference of two currents yields a BK component. In Figure 1A, BK + K_V and K_V currents were activated by two voltage steps to +80 mV with or without 0 mV prepulse from a holding potential of −60 mV, respectively. The prepulse to 0 mV was used to load extracellular Ca^{2+} ions into cells to

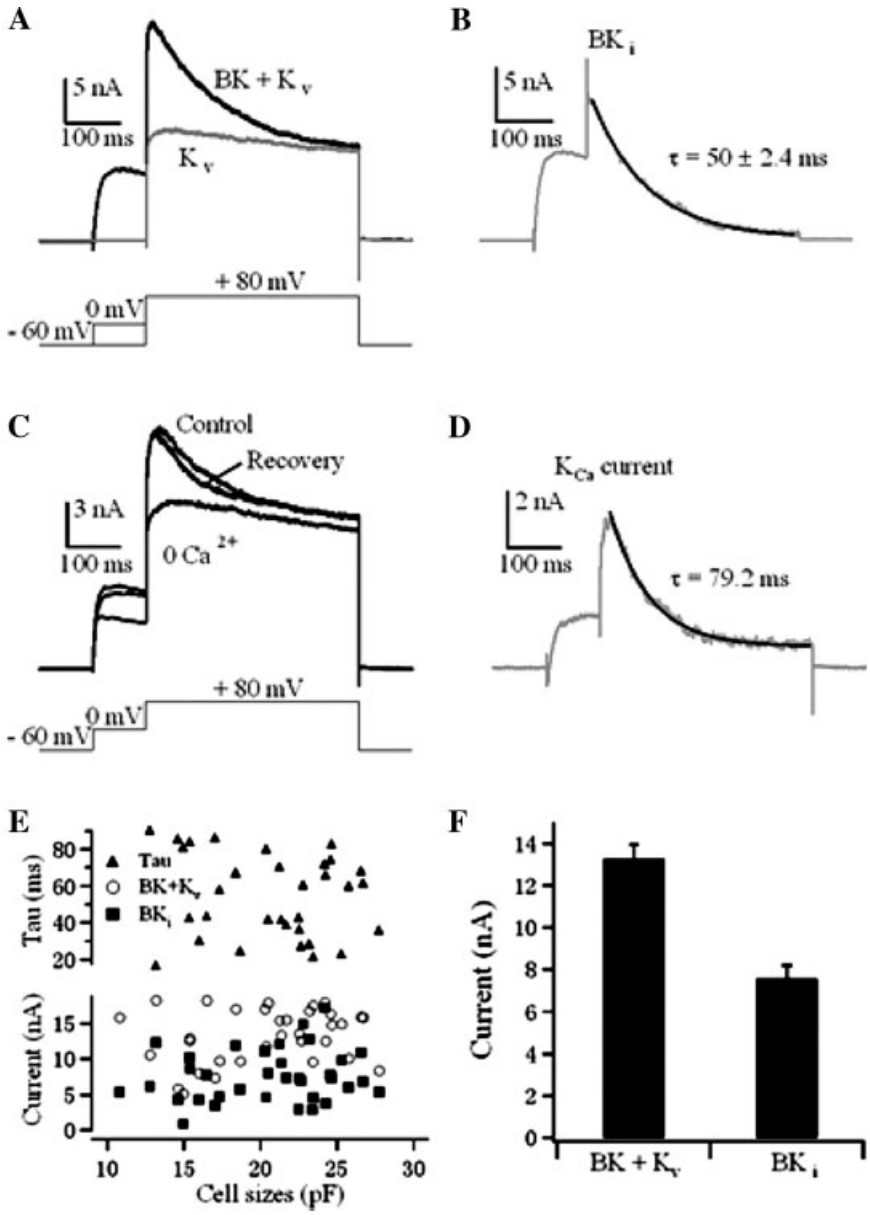

Fig. 1. Properties of the inactivating Ca^{2+}-dependent K^+ currents in DRG neurons. A: Typical traces from whole-cell recording were obtained from small DRG neurons. The cell was stepped from a holding potential of −60 mV to either −60 mV or 0 mV for 100 msec and then to +80 mV for 400 msec. Voltage protocol is shown at the bottom. Unless otherwise stated, cells were bathed in normal saline. The trace from a 0 mV prepulse contains both Ca^{2+}-dependent K^+ and K_V ($BK + K_V$) currents, but the trace from a −60 mV prepulse contains only of K_V current. B: The difference between the two traces gives a relative net Ca^{2+}-dependent K^+ (BK_i) current. A monoexponential fit of Ca^{2+}-dependent K^+ current gives a time constant of inactivation, $\tau_i = 50 \pm 2.4$ msec ($n = 32$). C: Application of calcium-free external solution reversibly eliminated the inactivating Ca^{2+}-dependent K^+ currents at +80 mV. D: The Ca^{2+}-sensitive K^+ current (K_{Ca} current) was obtained by subtracting the current in calcium-free external solution from the one in normal extracellular solution. The best monoexponential fit to the inactivating current gives a time constant of inactivation, $\tau_i = 79.2$ msec. E: Summary for inactivating currents of Ca^{2+}-dependent K^+ channels versus cell sizes. The time constants of inactivation or peak currents at +80 mV are plotted as function of cell sizes (pF). The symbols, solid triangle (▲), empty circle (○), and solid square (■), represent the time constant of BK_i, the peak current of $BK + K_V$ and the peak current of BK_i, respectively. F: Statistical mean peak currents of $BK + K_V$ and BK_i show that the BKi current is about 57.2% of total measured current.

activate BK currents. Their difference was thought to give a noninactivating BK current; however, our experiments show a distinct inactivating character (Fig. 1B). A mono-exponential fit to the BK_i currents yields an average inactivation time constant $\tau_i = 50 \pm 2.4$ msec ($n = 32$). To examine the Ca^{2+}-dependence of inactivating potassium currents, we changed the normal bath saline to Ca^{2+}-free saline by replacing 2 mM Ca^{2+} with 2 mM Mg^{2+} to minimize surface charge effects. Inactivating current was diminished greatly in presence of the Ca^{2+}-free external saline and resumed immediately after applying 2 mM Ca^{2+} external saline (Fig. 1C), suggesting that the current is sensitive to Ca^{2+} (K_{Ca} current). In Figure 1D, the K_{Ca} current

has an inactivation time constant of 79.2 msec at +80 mV. Twelve out of nineteen neurons exhibit identical inactivating behavior.

According to the classification of DRG neurons (Abdulla and Smith, 2001), DRG neurons with a capacitance larger than 90 pF, between 70 and 90 pF and less than 70 pF are defined as large, medium, and small neurons, respectively. Small DRG neurons were used in this study. From 82 DRG neurons, the mean value of capacitance per cell was 21.6 ± 1.0 pF, that is, a diameter of 26.2 ± 5.5 μm, assuming a spherical cell shape and a specific capacitance of 1 μF/cm^2 (Neher and Marty, 1982). Of all 82 DRG neurons, we found that there were 79 with inactivating BK currents, 2 with noninactivating BK currents, and 1 with no BK current (data not shown). Therefore, we infer that the inactivating BK channels are widely expressed in small DRG neurons, opposite to previous belief that BK_i channels are rare in neurons (Solaro and Lingle, 1992; Ding et al., 1998; Li et al., 1999). The time constants of inactivation derived from a mono-exponential fit to the BK_i currents are plotted as a function of neuron sizes (Fig. 1E top). To investigate whether the inactivating BK currents depended on cell size in small DRG neurons, the peak currents of $BK + K_V$ and BK_i are plotted versus cell sizes at the bottom of Figure 1E. There was no clear correlation between them to be found. The statistical mean peak currents of $BK + K_V$ and BK_i are shown in Figure 1F. The mean value of $BK + K_V$ and BK_i are 13.3 ± 0.7 nA and 7.6 ± 0.6 nA ($n = 35$). The BK_i currents are about 57.2% of total K^+ currents ($BK + K_V$), which may be underestimated due to many unknown factors such as inactivation numbers of BK_i channels, intracellular Ca^{2+} concentration and so on.

Blockade of charybdotoxin and iberiotoxin on the BK_i currents in rat DRG neurons

The BK channel blockers, IbTX (more specific than ChTX) and ChTX, were used at nanomolar concentration to characterize BK channels and to explore the physiological role of BK channels in vertebrates and mammalian (Meera et al., 2000b). The EC_{50} required for ChTX blocking inactivating BK channels is about 27.9 nM in RIN cell (Li et al., 1999) and 24.6 nM in rat adrenal chromaffin cells (Ding et al., 1998). Estimates of ChTX blockade for noninactivating BK channels from chromaffin cells and heterologously expressed BK channels yield an EC_{50} of 2 nM. These suggest that inactivating BK currents are much less sensitive to ChTX than typical noninactivating BK currents. Therefore, 100 nM ChTX and IbTX, we thought, were an appropriate concentration for completely blocking the inactivating BK currents in DRG neurons. In addition, 100 nM ChTX and IbTX were also used to study the function of BK channels in RIN cell (Li et al., 1999; Zhang et al., 2003). In this study, the BK channel blockers ChTX and IbTX were also used to confirm whether the aforementioned large-conductance, voltage- and Ca^{2+}-gated currents were indeed BK currents. Upon extracellular application of 100 nM ChTX to the DRG neurons for 2 min, the BK_i currents were, on average, reduced to $53.3 \pm 5.8\%$ ($n = 8$) (Fig. 2A). The current recovered from ChTX inhibition was incomplete ($75.8 \pm 4.2\%$, $n = 13$) after 5–6 min washing with normal saline (Fig. 2B), which is consistent with previous findings (Ding et al., 1998; Li et al., 1999). The BK_i currents in Figure 2A were blocked by 100 nM ChTX with an onset time constant $\tau_{on} = 44.7$ sec and an off-time constant $\tau_{off} = 295.1$ sec (Fig. 2C). The inhibitory effect of ChTX on BK_i channels was investigated by voltage steps for 400 msec ranging from −60 to +100 mV with 10 mV increments in the presence and absence of 100 nM ChTX (Fig. 2D). As shown in Figure 2E, inhibition is increased at more positive voltage. The same experiments were performed to evaluate the effect of 100 nM IbTX on the BK_i currents in DRG neurons (Fig. 2F). 100 nM IbTX blocked $41.3 \pm 5.7\%$ of BK_i currents ($n = 6$) with $59.7 \pm 8.8\%$ recovery ($n = 4$) after 5–6 min washing with normal saline (Fig. 2G). The BK_i currents in Figure 2F were blocked with an onset time constant $\tau_{on} = 46.4$ sec and an off-time constant $\tau_{off} = 251.6$ sec, (Fig. 2H). The above results reveal that the voltage- and Ca^{2+}-dependent currents in DRG sensory neurons are derived from BK_i channels because they are sensitive to both toxins ChTX and IbTX.

The rβ2-subunit exists in rat DRG neuron

Total RNA from the rat DRG was extracted and used as the RT-PCR template. The RT-PCR product amplified by primers was about 700 bps according to DNA electrophoresis, a length similar to rat BK β2 cDNA (Fig. 3A). The RT-PCR product was subcloned into pCR2.1-TOPO vector for DNA sequencing. The resulting sequence showed a very high homology to the rat β2 subunit of calcium-activated potassium channels (KCNMB2, PubMed NP_789831.1) and rβ2 cloned in RINm5f cells (Xia et al., 1999), with the exception of three amino acids (Fig. 3B). We suggest that R84 in the KCNMB2 PubMed sequence, which differs from one of four conserved cysteines, C84, in the rβ2 of DRG and RINm5f cells, may reflect a genetic difference between different rat strains. We propose that the rat BK β2-subunit (rβ2) cloned in this study is present in rat DRG neurons and that it is responsible for the inactivation of BK channels in these cells. Four β-subunits in all have been found from the human EST database, two of which, β2 and β3, show inactivation (Wallner et al., 1999; Xia et al., 1999, 2000). Since BK channels exhibited a fast inactivation with $\tau_i \sim 1$–4 msec in DRG cells consistently, we attempted to detect the β3 subunit in DRG cells on the basis of the hβ3-subunit sequence. However, we failed to amplify any β3-subunit sequences by RT-PCR with a similar protocol (data not shown) and inferred that expressed rβ3-subunits are absent, or they are presented too low to be detectable in DRG neurons.

The kinetic characteristics of BK_i channels encoded by mSlo1 α- and rβ2-subunits in the rat small diameter DRG neurons

Calcium dependence is a basic characteristic of BK channels. Traces recorded from an inside-out patch, at 10 μM Ca^{2+} intracellular solution, show the typical characteristics of the inactivating BK currents presented in the left five parts in Figure 4A. The single BK_i channel currents were diminished after applying 0 μM Ca^{2+} solution ($n = 6$) shown at rightmost column in Figure 4A. Their ensemble currents are shown at the bottom. Inactivation time constants of BK_i channels in DRG neurons are shown in Figure 4B ($n = 6$). The conductance of the BK channel ranges from 150 to 300 pS in symmetrical K^+ solutions depending on the tissue type (Latorre et al., 1989; McManus and Magleby, 1991; Vergara et al., 1998). In Figure 4C, a set of representative traces containing one BK_i channel in an inside-out patch, exhibits transient single-channel openings at different potentials in the presence of 10 μM Ca^{2+}. The mean single-channel conductance of BK_i channel is 217.1 ± 1.2 pS for outward currents ranged from +20 mV to +100 mV and 91.6 ± 0.2 pS for inward currents ranged from −100 mV to −20 mV ($n = 8$, Fig. 4D). This compares with the conductance (250–260 pS) of cloned BK channels co-expressed by mSlo1 α- and hβ2-subunits (Xia et al., 1999). The BK_i channel conductance in rat DRG neurons is about 220 pS, slightly smaller than the 250 pS of cloned hβ2 genes due perhaps to the genetic difference between the rβ2 and hβ2. However, BK_i channels in rat DRG cells show rectification as the co-expressed mSlo1/hβ2 channels does (Zeng et al., 2003). The BK_i channel in rat chromaffin cells is composed of mSlo1 α-subunits with up to four rβ2-subunits per channel (Ding et al., 1998; Wang et al., 2002). With 160 mM symmetrical K^+

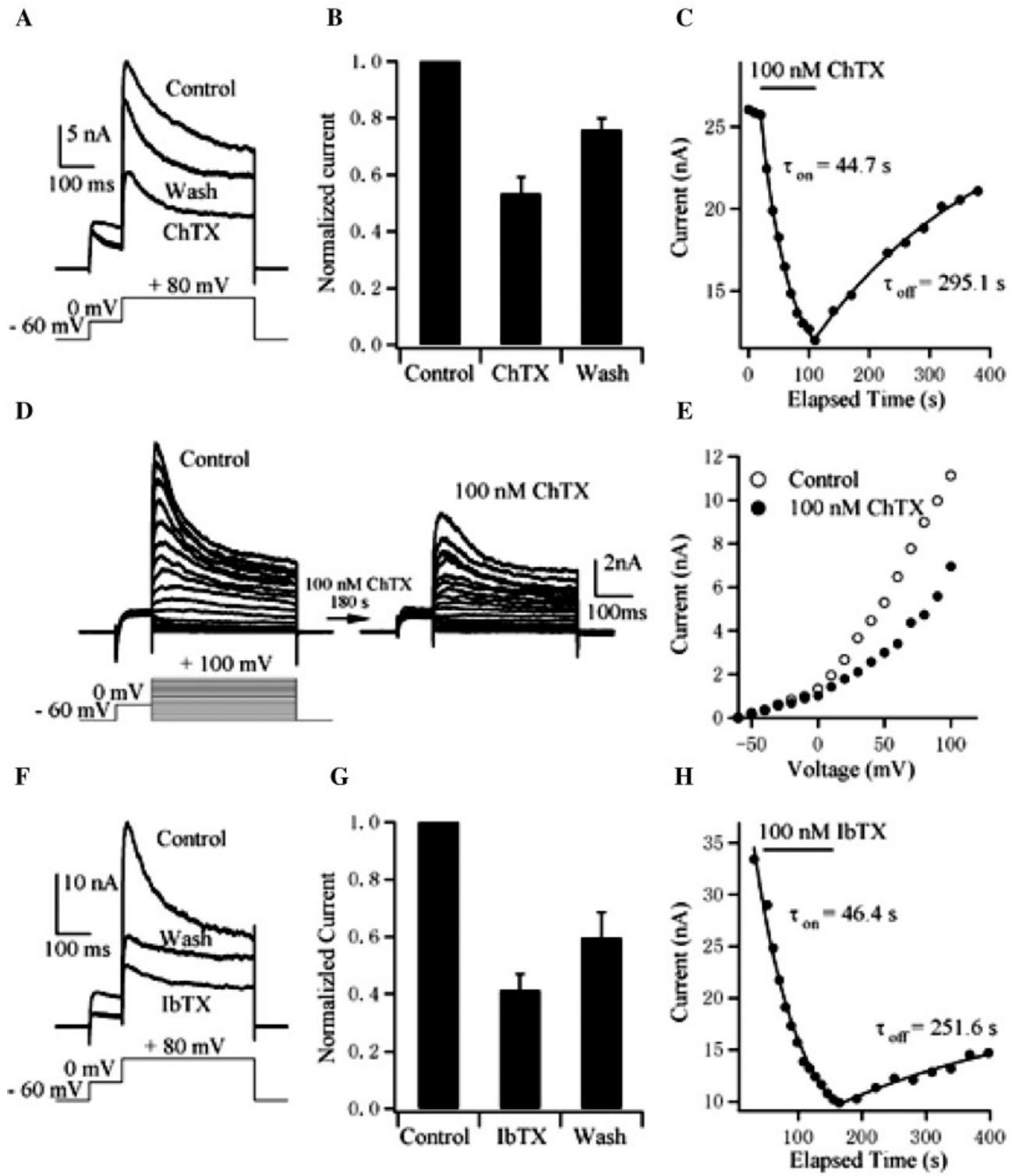

Fig. 2. Both charybdotoxin and iberiotoxin block the currents of BK_i channels in the rat small-diameter DRG neurons. A: BK_i currents activated by a voltage step to +80 mV before, during, and after the application of 100 nM charybdotoxin (ChTX) are shown. The voltage protocol is shown below. Each cell was held at −60 mV and stepped to 0 mV for 100 msec and then stepped directly to +80 mV for 400 msec. Obviously, recovery of BK_i channels from inhibition by toxins is incomplete. B: The normalized inhibition and recovery of BK_i channels from blockade by 100 nM ChTX are 53.3 ± 5.8% (n = 8) and 75.8 ± 4.2% (n = 13), respectively. C: Amplitudes of peak currents as a function of elapsed time for the cell shown in A are plotted along with two fits of single exponential function with an on-time constant τ_{on} = 44.7 sec and an off-time constant τ_{off} = 295.1 sec. Perfused with 100 nM ChTX is indicated by the horizontal bar. D: Cell was held at −60 mV and stepped from a holding potential to 0 mV for 100 msec and then stepped directly to potentials ranging from −60 to +100 mV with 10 mV increments for 400 msec, in the presence or absence of 100 nM ChTX (horizontal arrow, for 180 sec). The voltage protocol is shown below the traces. Compared with traces on the left, the outward current traces on the right are clearly reduced by 100 nM ChTX, a blocker of BK channels. E: The peak currents of the cell are plotted as a function of membrane potentials before (○) and during (●) application of 100 nM ChTX. In F,G,H, similar inhibition resulted from 100 nM iberiotoxin (IbTX), giving a τ_{on} = 46.4 sec and τ_{off} = 251.6 sec. Normalized inhibition and recovery of BK_i channels by 100 nM IbTX are 41.3 ± 5.7% (n = 6) and 59.7 ± 8.8% (n = 4), respectively.

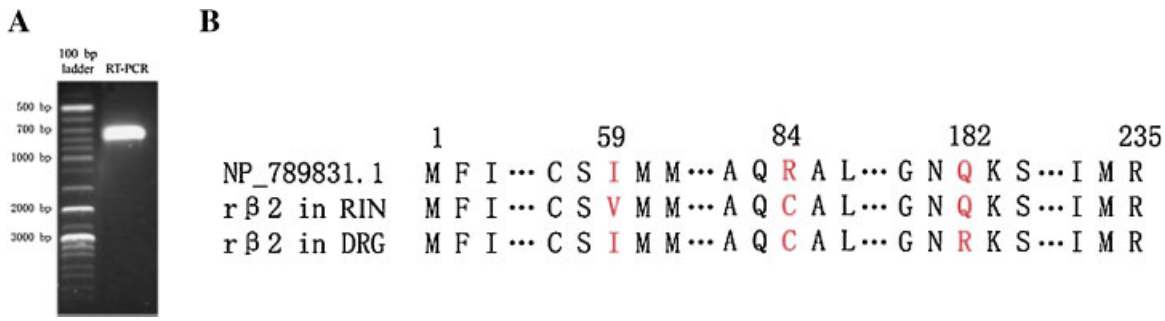

Fig. 3. Sequence analysis of the rβ2-subunit in the rat DRG neurons by RT-PCR. A: Total RNA was extracted from rat DRG neurons and used as a RT-PCR template. By the DNA electrophoresis the product of RT-PCR is a cDNA with a length about 700 bps, similar to the rβ2 subunit of BK channels. B: The resulting sequence shares a high homology to the rat calcium-activated potassium channel β2 subunit (KCNMB2, PubMed NP_789831.1) and rβ2 cloned in RINm5f cells, except for three amino acids labeled in red. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

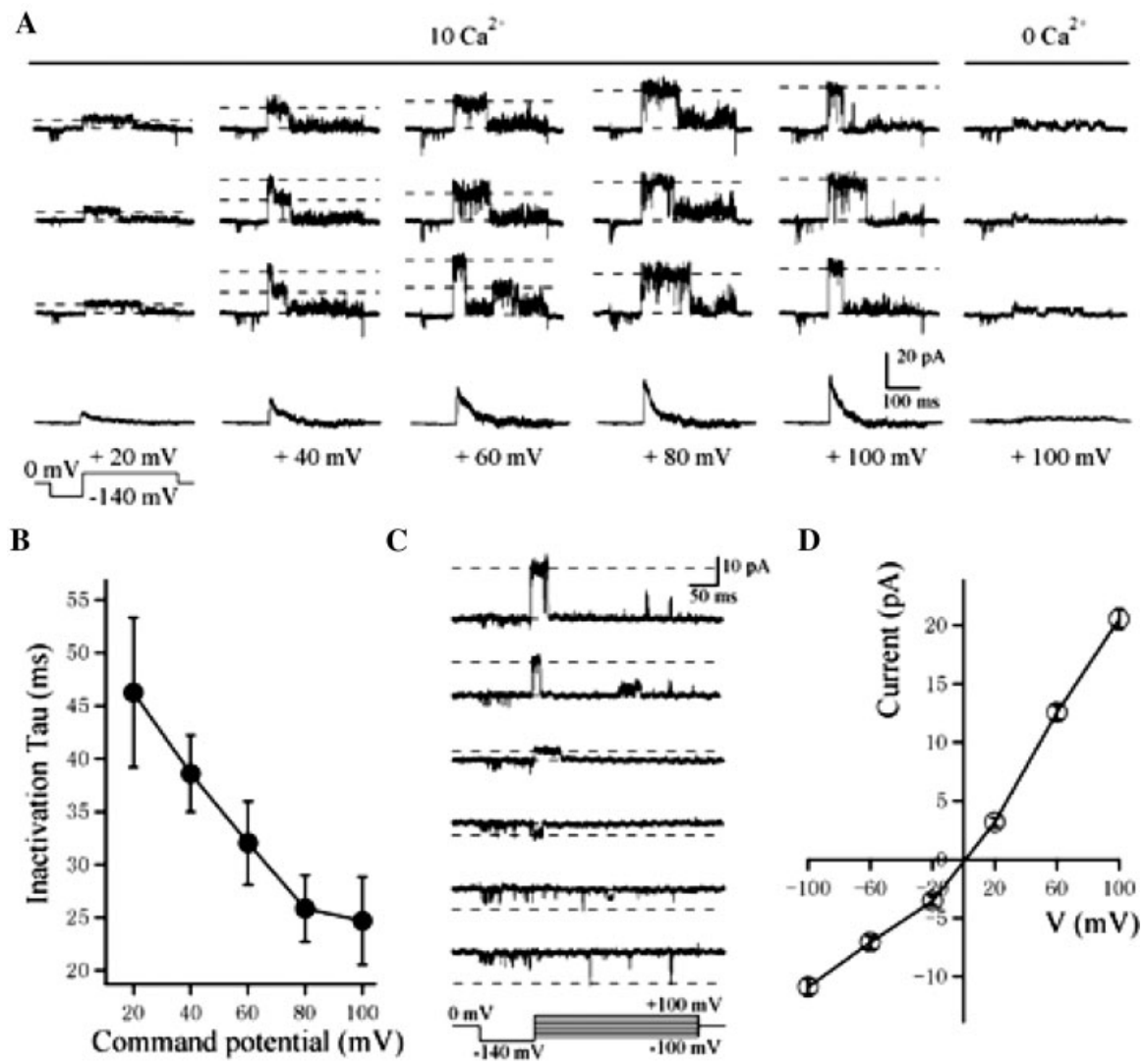

Fig. 4. Kinetic characteristics of BK_i channels in the small DRG neurons. A: Representative traces containing two BK channels were recorded from an inside-out patch with symmetrical 160 mM K^+ solutions. Currents were activated by voltage steps from +20 to +100 mV from a holding potential of −140 mV with 10 μM Ca^{2+}. The inactivating BK channels were distinctly sensitive to cytoplasmic 10 μM Ca^{2+}. The channel are absent with 0 μM Ca^{2+} at +100 mV. In each part, the top three traces show examples of records of single-channel openings at five different potentials, whereas the bottom trace shows the ensemble currents at each potential. For each single channel trace, the dashed lines indicate the open current level (top) and closed current level (bottom). B: The inactivation time constants of the ensemble currents are plotted at different command potentials. C: Single-channel conductance was recorded from −100 to +100 mV from a holding potential at −140 mV in 40 mV increments with 10 μM Ca^{2+}. D: I–V relationship of the single BK channel is plotted. The mean outward conductance of inactivating BK channels is 217.1 pS (n = 8).

solutions, a BK channel assembled with 1–4 rβ2-subunits is expected to have an inactivation time constants of 80, 40, 27, and 20 msec at +100 mV, respectively in the presence of 10 μM intracellular Ca^{2+} (Ding et al., 1998; Wang et al., 2002). We found that BK_i inactivation time constants ranging from 15 to 35 msec at +100 mV with an average $\tau_I = 24.7 \pm 4.2$ msec ($n = 6$) in Figure 4B. Considering these measurements of the inactivation kinetics and single channel conductance of BK_i channels, we infer that the average stoichiometry is three rβ2-subunits per channel in small DRG sensory neurons.

Trypsin removes inactivation of BK_i channels in DRG neurons

N-type inactivation of ion channels can be interpreted with a so-called "ball-and-chain" mechanism proposed for the inactivation of Na^+ channels (Armstrong and Bezanilla, 1977). Following channel opening, according to this model, a positively charged particle (the "ball") on a tether (the "chain") near the pore of the ion channel, blocks ionic conduction by occluding the pore. Removing inactivation with protease (commonly trypsin) is a typical way to demonstrate N-type inactivation. Trypsin attacks most of ten basic residues in the N-terminus (Xia et al., 1999), especially the residues near the N-terminus of hβ2 subunit, for example, R8, R14, K18, and R19. To demonstrate N-type inactivation, trypsin is transiently applied to the intracellular face of channels to cleave the inactivation ball (or the chain) and thereby remove inactivation. In rat chromaffin cells, a brief exposure to trypsin removes the inactivation of BK_i channels, suggesting that it is an N-type inactivation mechanism in these channels (Solaro and Lingle, 1992; Ding et al., 1998). Therefore, 0.5 mg/ml trypsin was applied to the intracellular face of BK_i channels to remove the inactivation of BK channels in rat DRG cells. Figure 5A shows representative inactivating single-channel currents from an inside-out patch activated by depolarizing to +100 mV from holding potential of −140 mV in the presence of 10 μM Ca^{2+}. Typical inactivating BK currents before and after applying trypsin are shown in Figure 5A. The assembled currents derived from 25 consecutive traces are shown in Figure 5B. Briefly applying trypsin to the cytosolic face within 3 min induced a gradual decrease in inactivation rate and a gradual augmentation in the steady-state levels of current (Fig. 5A,B). After 3 min application with trypsin, BK_i channels became noninactivating, suggesting that this is the same N-type inactivation found in chromaffin cells. Six neurons were tested in this experiment.

BK_i channels participate in modulating the action potentials (AP) in DRG neurons

To evaluate the physiological significance of the BK_i channels identified in DRG sensory neurons, we examined the involvement of BK_i in shaping the APs in DRG neurons. In the current-clamp recording, APs were initiated by injecting 0.45 nA current. Typically, this current injection triggers only one AP. IbTX, a specific blocker of BK channels, was used to block BK_i channels in current-clamp condition. IbTX (100 nM) distinctly increased the firing frequency (Fig. 6A) and suppressed the AHP (Fig. 6B). With the application of 100 nM IbTX, the value of AHP was reduced by 16.9 ± 4.5 mV ($n = 6$), and the width of single AP at half-maximal amplitude was broadened by $11.8 \pm 0.3\%$ ($n = 8$) (Fig. 6B). From Figure 6C, the derivative of APs shown in Figure 6B reveals that IbTX reduce the AHP. As a consequence, BK_i channels may reduce calcium influx during APs. These results suggest that opening BK_i channels may play an important role in suppressing the hyperexcitability of sensory neurons in physiological and pathological states (Faber and Sah, 2003).

Discussion

In this study, we have investigated the inactivating characteristics, pharmacology and function of BK_i channels in small neurons taken from lumbar L4-6 DRG of young adult Wistar rats. The results indicate that the inactivating currents of BK_i channels in DRG neurons are mainly caused by auxiliary rβ2-subunits, contrary to previous studies (Naruse et al., 1992; Gruss et al., 2001; Zhang et al., 2003). The evidence for BK_i channels existing in DRG neurons are as following: firstly, inactivating currents were recorded from both whole-cell (Figs. 1 and 2) and single-channel recordings (Fig. 4); secondly, the sequence extracted from rat DRG neurons shows a very

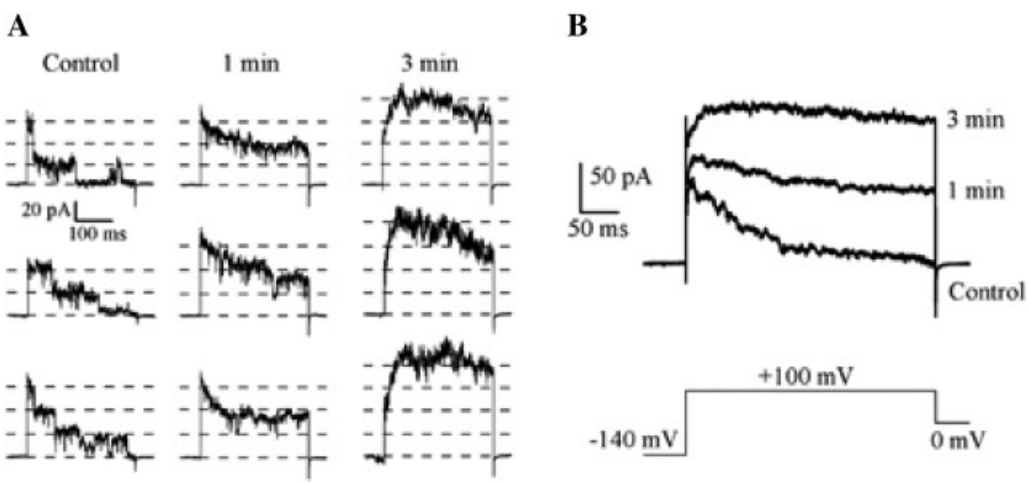

Fig. 5. Trypsin eliminates inactivation of BK_i channels. A: Currents collected from an inside-out patch in the present of 10 μM Ca^{2+} from a DRG neuron, activated by a voltage step to +100 mV, after a prepulse to −140 mV to remove inactivation. The representative traces were shown before (Control) and after applying with 0.5 mg/ml trypsin for 1 and 3 min. The dashed lines indicate a close (bottom) and 2–5 open levels (top) in currents. B: Average currents of 20–30 traces displayed as time elapsed after intracellularly applying 0.5 mg/ml trypsin. Obviously, trypsin gradually removes the inactivation of BK_i channels ($n = 6$). The voltage protocol is shown at the bottom.

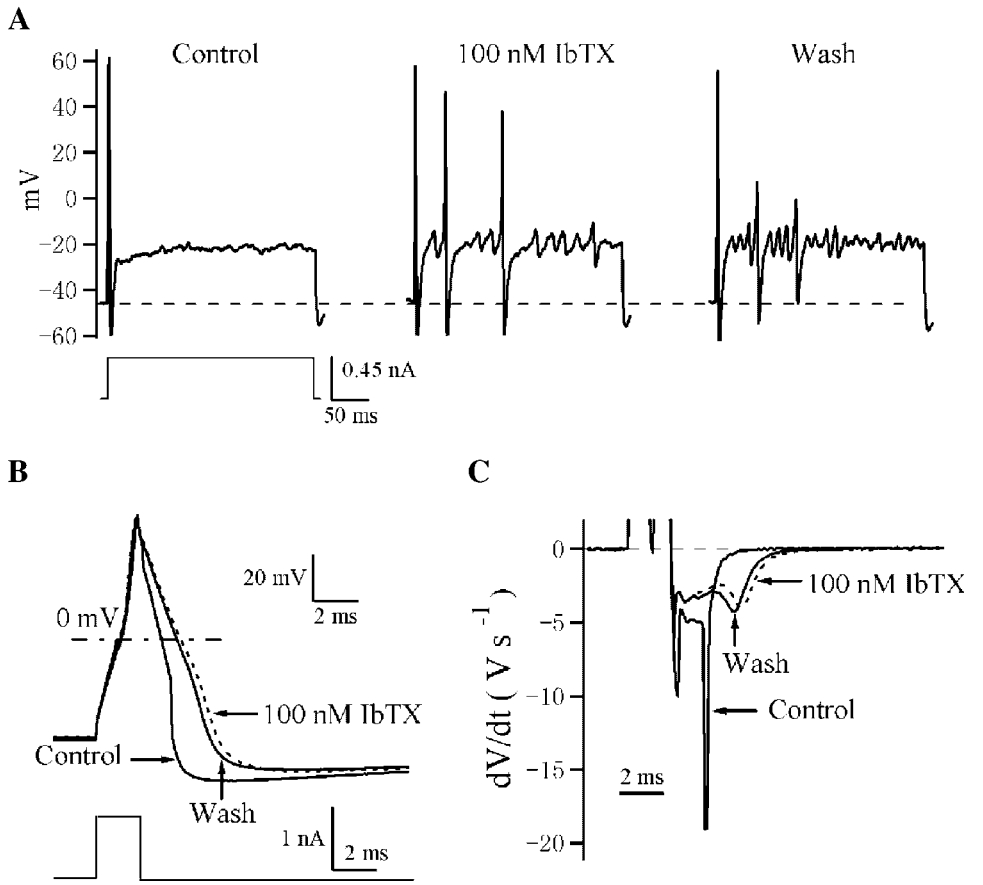

Fig. 6. BK_i channels modulate DRG action potentials. A: In current-clamp recording mode, a cell was depolarized to fire one action potential by injecting 0.45 nA current from a resting potential. Blockade of BK_i channels with 100 nM IbTX increases the firing frequency under identical conditions. B: Application of 100 nM IbTX (dashed line) broadens the duration and reduces the after-hyperpolarization (AHP), in a single action potential evoked by injecting 1 nA current. C: The derivatives of action potentials shown in B are plotted.

high homology to the sequences of KCNMB2 and rβ2 from RINm5f cells (Xia et al., 1999) (Fig. 3); thirdly, the inactivation of BK_i channels can be removed by trypsin (0.5 mg/ml) applied on the cytoplasmic side (Fig. 5). Moreover, our pharmacological experiments indicate that the inactivating BK_i channels are sensitive to both BK channel blockers, ChTX and IbTX (Fig. 2). Except for heart myocytes, BK channels with their β-subunits are almost ubiquitously expressed among mammalian tissues and play a variety of roles (Latorre et al., 1989; McManus and Magleby, 1991; Solaro and Lingle, 1992; Li et al., 1999; Xia et al., 1999, 2000; Brenner et al., 2000; Ramanathan and Fuchs, 2002). BK currents exhibit a tremendous range of properties in ion-dependency, single-channel conductance and inactivation from cell to cell. The origin of this functional channel diversity arises from the combination of the Slo1 α-subunits with four members of auxiliary (-subunits, and alternative splicing of the Slo mRNA (Adelman et al., 1992; Tseng-Crank et al., 1994; Saito et al., 1997; Ramanathan et al., 1999). Only a few native BK channels have been defined in detail and extensively studied due to their wide distribution and great diversity. To our knowledge, the inactivating BK_i currents have been observed in several cell types including skeletal muscle (Pallotta, 1985), hippocampal neurons (Ikemoto et al., 1989; Hicks and Marrion, 1998), pancreatic β cells (Li et al., 1999) and chromaffin cells (Lingle et al., 1996). Only rarely do BK channels exhibit inactivation molecular determinants, especially in neurons. In this study, we have examined in detail the properties of BK_i channels in DRG neurons using whole-cell recordings and inside-out patches. In both recording modes, BK channels in DRG neurons exhibited distinct inactivation characteristics in 96% of the cells (n = 80/83). This raises a question why three previous studies failed to detect BK_i channels in DRG neurons (Naruse et al., 1992; Gruss et al., 2001; Zhang et al., 2003). One of the reasons could be a difference in digestion procedures used. The possibility that BK_i channels are susceptible to extracellular enzyme treatment (Armstrong and Roberts, 2001) implies that BK_i channels may be present in many other cell types. Another possible explanation for this discrepancy might be that we only collected DRG from lumbar L4-6 segments from young adult rats. It would be interesting to compare the spatial and temporal expression profile of BK_i channels in DRG neurons.

The regulatory β-subunits associated with Slo1 can cause dramatic changes in Ca^{2+}-dependence, kinetics and pharmacological characteristics of BK_i currents (McManus et al., 1995; Wallner et al., 1995, 1999; Xia et al., 1999, 2000). In total, four β-subunit genes, that is, hβ1–hβ4, have been found so far from the human genome (Meera et al., 2000a). In rats, only β1, β2, and β4 have been identified, whereas β3 remains unknown (Weiger et al., 1998; Jiang et al., 1999; Xia et al., 1999) and only β2 and β3 of the β-family exhibited inactivation. Armstrong (Armstrong and Roberts, 2001) has claimed that a β subunit similar to β2 (maybe β3) is found in frog saccular hair cells, and Sun (Sun et al., 2003) found β2 in mouse neocortical

pyramidal neurons. The kinetics of native inactivating BK currents in both cell types is clearly not consistent with data from the cloned Slo1/hβ3b complex (Xia et al., 2000). In some experiments, the BK_i channels in DRG neurons showed a very rapid inactivation with a time constant ranging from 1 to 8 msec along with a slow component. However, we could not distinguish it from capacitance due to its very rapid inactivation and also failed to validate it in DRG cells by RT-PCR. The rat BK-type β3 (PubMed XM_227040) is predicted from automated computational analysis. Based on the hβ3-sequence and a RT-PCR protocol similar that used to detect rβ2′ sequences, three pairs of primers were designed to detect the rβ3-subunit, but all of them failed (data not shown). Considering that an rβ3 may significantly differ from the hβ3, we still cannot exclude the possibility that a novel rβ3 gene may exist in DRG neurons. The small-diameter DRG neurons are closely related to pain-conducting Aδ- and C-fibers (Harper and Lawson, 1985a,b). In this study, we thus restricted our investigation on the small DRG neurons from lumbar ganglia L4 to L6, where sciatic DRG neurons are normally located (Swett et al., 1991). We demonstrate that blockade of BK_i channels can prolong the repolarization phase and increase the firing frequency in DRG neurons. Together with previous findings (Pedarzani et al., 2000; Zhang et al., 2003), our results suggest that BK_i channels are likely involved in modulating nociceptive information from the peripheral to the central nervous system (CNS). During prolong current injections, the modulation of the fast AHP by BK_i channels will become more significant. Interestingly, the activation of BK_i channels induced by clinically relevant concentrations of ethanol results in modulation of AP duration (APD) and refractory period of DRG neurons, which might contribute to the well-known ethanol-induced analgesia and paresthesia (Gruss et al., 2001). The β2-subunits have been reported to negatively shift the G–V curve to increase the activity of BK channels (Xia et al., 1999), suggesting that rβ2-subunits facilitate AHP in DRG cells through this mechanism. However, in the case of high Ca^{2+} concentration, inactivation induced by rβ2-subunits may prevent the BK currents in DRG cells from over-activating so as to maintain a regular firing of APs, which may contribute to sensing pain. Consequently, the presence of rβ2-subunits in small DRG neurons should play an important role in modulating the conduction of information from periphery to the CNS.

Acknowledgments

We thank Dr. A. Ward for his critical comments. The laboratory of TX belongs to a Partner Group Scheme of the Max Planck Institute for Biophysical Chemistry, Göttingen.

Literature Cited

Abdulla FA, Smith PA. 2001. Axotomy- and autotomy-induced changes in the excitability of rat dorsal root ganglion neurons. J Neurophysiol 85:630–643.
Adelman JP, Shen KZ, Kavanaugh MP, Warren RA, Wu YN, Lagrutta A, Bond CT, North RA. 1992. Calcium-activated potassium channels expressed from cloned complementary DNAs. Neuron 9:209–216.
Armstrong CM, Bezanilla F. 1977. Inactivation of sodium channel II gating currents experiments. J Gen Physiol 70:567–590.
Armstrong CE, Roberts WM. 2001. Rapidly inactivating and non-inactivating calcium-activated potassium currents in frog saccular hair cells. J Physiol 536:49–65.
Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. 2000. Vasoregulation by the β1 subunit of the calcium-activated potassium channel. Nature 407:870–876.
Devor M. 1999. Unexplained peculiarities of the dorsal root ganglion. Pain Suppl 6:S27–S35.
Ding JP, Li ZW, Lingle CJ. 1998. Inactivating BK channels in rat chromaffin cells may arise from heteromultimeric assembly of distinct inactivation-competent and noninactivating subunits. Biophys J 74:268–289.
Dworetzky SI, Boissard CG, Lum-Ragan JT, McKay MC, Post-Munson DJ, Trojnacki JT, Chang CP, Gribkoff VK. 1996. Phenotypic alteration of a human BK (hSlo) channel by hSlo β subunit coexpression: Changes in blocker sensitivity, activation/relaxation and inactivation kinetics, and protein kinase A modulation. J Neurosci 16:4543–4550.
Faber ES, Sah P. 2003. Ca^{2+}-activated K^+ (BK) channel inactivation contributes to spike broadening during repetitive firing in the rat lateral amygdala. J Physiol 552:483–497.
Goodman MB, Art JJ. 1996. Variations in the ensemble of potassium currents underlying resonance in turtle hair cells. J Physiol 497:395–412.
Gruss M, Henrich M, Konig P, Hempelmann G, Vogel W, Scholz A. 2001. Ethanol reduces excitability in a subgroup of primary sensory neurons by activation of BK(Ca) channels. Eur J Neurosci 14:1246–1256.
Harper AA, Lawson SN. 1985a. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. J Physiol 359:31–46.
Harper AA, Lawson SN. 1985b. Electrical properties of rat dorsal root ganglion neurones with different peripheral nerve conduction velocities. J Physiol 359:47–63.
Hicks GA, Marrion NV. 1998. Ca^{2+}-dependent inactivation of large-conductance Ca^{2+}-activated K^+ (BK) channels in rat hippocampal neurones produced by pore block from an associated particle. J Physiol 508:721–734.
Ikemoto Y, Ono K, Yoshida A, Akaike N. 1989. Delayed activation of large-conductance Ca^{2+}-activated K^+ channels in hippocampal neurons of the rat. Biophys J 56:207–212.
Jiang Z, Wallner M, Meera P, Toro L. 1999. Human and rodent MaxiK channel β-subunit genes: Cloning and characterization. Genomics 55:57–67.
Latorre R, Oberhauser A, Labarca P, Alvarez O. 1989. Varieties of calcium-activated potassium channels. Annu Rev Physiol 51:385–399.
Li ZW, Ding JP, Kalyanaraman V, Lingle CJ. 1999. RINm5f cells express inactivating BK channels whereas HIT cells express noninactivating BK channels. J Neurophysiol 81:611–624.
Lingle CJ, Solaro CR, Prakriya M, Ding JP. 1996. Calcium-activated potassium channels in adrenal chromaffin cells. Ion Channels 4:261–301.
McManus OB, Magleby KL. 1991. Accounting for the Ca^{2+}-dependent kinetics of single large-conductance Ca^{2+}-activated K^+ channels in rat skeletal muscle. J Physiol 443:739–777.
McManus OB, Helms LM, Pallanck L, Ganetzky B, Swanson R, Leonard RJ. 1995. Functional role of the β subunit of high conductance calcium-activated potassium channels. Neuron 14:645–650.
Meera P, Wallner M, Toro L. 2000a. A neuronal β subunit (KCNMB4) makes the large-conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. Proc Natl Acad Sci USA 97:5562–5567.
Meera P, Wallner M, Toro L. 2000b. A neuronal β subunit (KCNMB4) makes the large-conductance, voltage- and Ca^{2+}-activated K^+ channel resistant to charybdotoxin and iberiotoxin. Proc Natl Acad Sci USA 97:5562–5567.
Naruse K, McGehee DS, Oxford GS. 1992. Differential responses of Ca^{2+}-activated K^+ channels to bradykinin in sensory neurons and F-11 cells. Am J Physiol 262:C453–C460.
Neely A, Lingle CJ. 1992. Two components of calcium-activated potassium current in rat adrenal chromaffin cells. J Physiol 453:97–131.
Neher E, Marty A. 1982. Discrete changes of cell membrane capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. Proc Natl Acad Sci USA 79:6712–6716.
Nimigean CM, Magleby KL. 1999. The β subunit increases the Ca^{2+} sensitivity of large-conductance Ca^{2+}-activated potassium channels by retaining the gating in the bursting states. J Gen Physiol 113:425–440.
Pallotta BS. 1985. Calcium-activated potassium channels in rat muscle inactivate from a short-duration open state. J Physiol 363:501–516.
Pedarzani P, Kulik A, Muller M, Ballanyi K, Stocker M. 2000. Molecular determinants of Ca^{2+}-dependent K^+ channel function in rat dorsal vagal neurones. J Physiol 527:283–290.
Ramanathan K, Fuchs PA. 2002. Modeling hair cell tuning by expression gradients of potassium channel β subunits. Biophys J 82:64–75.
Ramanathan K, Michael TH, Jiang GJ, Hiel H, Fuchs PA. 1999. A molecular mechanism for electrical tuning of cochlear hair cells. Science 283:215–217.
Rasband MN, Park EW, Vanderah TW, Lai J, Porreca F, Trimmer JS. 2001. Distinct potassium channels on pain-sensing neurons. Proc Natl Acad Sci USA 98:13373–13378.
Saito M, Nelson C, Salkoff L, Lingle CJ. 1997. A cysteine-rich domain defined by a novel exon in a slo variant in rat adrenal chromaffin cells and PC12 cells. J Biol Chem 272:11710–11717.
Scholz A, Gruß M, Vogel W. 1998. Properties and functions of calcium-activated K^+ channels in small neurones of rat dorsal root ganglion studied in a thin slice preparation. J Physiol 5131:55–69.
Solaro CR, Lingle CJ. 1992. Trypsin-sensitive, rapid inactivation of a calcium-activated potassium channel. Science 257:1694–1698.
Solaro CR, Prakriya M, Ding JP, Lingle CJ. 1995. Inactivating and noninactivating Ca^{2+}- and voltage-dependent K^+ current in rat adrenal chromaffin cells. J Neurosci 15:6110–6123.
Sun X, Gu XQ, Haddad GG. 2003. Calcium influx via L- and N-type calcium channels activates a transient large-conductance Ca^{2+}-activated K^+ current in mouse neocortical pyramidal neurons. J Neurosci 23:3639–3648.
Swett JE, Torigoe Y, Elie VR, Bourassa CM, Miller PG. 1991. Sensory neurons of the rat sciatic nerve. Exp Neurol 114:82–103.
Tseng-Crank J, Foster CD, Krause JD, Mertz R, Godinot N, DiChiara TJ, Reinhart PH. 1994. Cloning, expression, and distribution of functionally distinct Ca^{2+}-activated K^+ channel isoforms from human brain. Neuron 13:1315–1330.
Tseng-Crank J, Godinot N, Johansen TE, Ahring PK, Strobaek D, Mertz R, Foster CD, Olesen SP, Reinhart PH. 1996. Cloning, expression, and distribution of a Ca^{2+}-activated K^+ channel β-subunit from human brain. Proc Natl Acad Sci USA 93:9200–9205.
Uebele VN, Lagrutta A, Wade T, Figueroa DJ, Liu Y, McKenna E, Austin CP, Bennett PB, Swanson R. 2000. Cloning and functional expression of two families of β-subunits of the large-conductance calcium-activated K^+ channel. J Biol Chem 275:23211–23218.
Vergara C, Latorre R, Marrion NV, Adelman JP. 1998. Calcium-activated potassium channels. Curr Opin Neurobiol 8:321–329.
Wallner M, Meera P, Ottolia M, Kaczorowski GJ, Latorre R, Garcia ML, Stefani E, Toro L. 1995. Characterization of and modulation by a β-subunit of a human maxi K_{Ca} channel cloned from myometrium. Receptors Channels 3:185–199.
Wallner M, Meera P, Toro L. 1999. Molecular basis of fast inactivation in voltage and Ca^{2+}-activated K^+ channels: A transmembrane β-subunit homolog. Proc Natl Acad Sci USA 96:4137–4142.
Wang YW, Ding JP, Xia XM, Lingle CJ. 2002. Consequences of the stoichiometry of Slo1 α and auxiliary β subunits on functional properties of large-conductance Ca^{2+}-activated K^+ channels. J Neurosci 22:1550–1561.
Weiger TM, Langer T, Hermann A. 1998. External action of di- and polyamines on maxi calcium-activated potassium channels: An electrophysiological and molecular modeling study. Biophys J 74:722–730.
Xia XM, Ding JP, Lingle CJ. 1999. Molecular basis for the inactivation of Ca^{2+}- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. J Neurosci 19:5255–5264.

Xia XM, Ding JP, Zeng XH, Duan KL, Lingle CJ. 2000. Rectification and rapid activation at low Ca^{2+} of Ca^{2+}-activated, voltage-dependent BK currents: Consequences of rapid inactivation by a novel β subunit. J Neurosci 20:4890–4903.

Xia XM, Ding JP, Lingle CJ. 2003. Inactivation of BK channels by the NH_2 terminus of the β2 auxiliary subunit: An essential role of a terminal peptide segment of three hydrophobic residues. J Gen Physiol 121:125–148.

Zeng XH, Xia XM, Lingle CJ. 2003. Redox-sensitive extracellular gates formed by auxiliary β subunits of calcium-activated potassium channels. Nat Struct Biol 10: 448–454.

Zhang XF, Gopalakrishnan M, Shieh CC. 2003. Modulation of action potential firing by iberiotoxin and NS1619 in rat dorsal root ganglion neurons. Neuroscience 122:1003–1011.