Manipulation of the potassium channel Kv1.1 and its effect on neuronal excitability in rat sensory neurons

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Running title: Kv1.1 and excitability in sensory neurons
ABSTRACT

Potassium channels play a critical role in regulating many aspects of action potential firing. To establish the contribution of the voltage-dependent potassium channel Kv1.1 in regulating excitability, we used the selective blocker, dendrotoxin-K (DTX-K) and siRNA targeted to Kv1.1 to determine their effects on action potential (AP) firing in small diameter capsaicin-sensitive sensory neurons. A 5 min exposure to 10 nM DTX-K suppressed the total potassium current ($I_K$) measured at +40 mV by approximately 33%. DTX-K produced a two-fold increase in the number of APs evoked by a ramp of depolarizing current. Associated with increased firing was a decrease in firing threshold and rheobase. DTX-K did not alter the resting membrane potential or the AP duration. A 48 hr treatment with siRNA targeted to Kv1.1 reduced the expression of this channel protein by ~60% as measured in western blots. After treatment with siRNA, $I_K$ was no longer sensitive to DTX-K, indicating a loss of functional protein. Similarly, after siRNA treatment exposure to DTX-K had no affect on the number of evoked APs, firing threshold, or rheobase. However, after siRNA treatment, the firing threshold had values that was similar to those obtained after acute exposure to DTX-K, suggesting that the loss of Kv1.1 plays a critical role in setting this parameter of excitability. These results demonstrate that Kv1.1 plays an important role in limiting AP firing and that siRNA may be a useful approach to establish the role of specific ion channels in the absence of selective antagonists.

Key Words: action potential, potassium current, sensitization, dendrotoxin-K, siRNA
INTRODUCTION

Voltage-gated potassium channels (Kv) are protein complexes consisting of ion-conducting integral protein α subunits and auxiliary cytoplasmic β subunits. These Kv channels are quite diverse due to numerous gene families, heteromultimeric combination of α subunits, auxiliary subunits, splice variants, and post-translational processing. More than a dozen α subunits of the Kv superfamily have been isolated and have been divided into several subfamilies, Kv1-6, 8, and 9 (Chandy and Gutman 1995; Gutman et al. 2003, 2005; Pongs 1992). Functionally, Kv channels play an important role in setting the resting membrane potential, in controlling repolarization of the action potential (AP), and in modulating the frequency of firing. However, the contribution that a specific channel subtype of the Kv family makes to a particular physiological aspect of neuronal membrane properties or AP firing is poorly understood because of the co-expression of multiple subtypes in any given neuron, the capacity of the α subunits to form heteromultimers, and the paucity of specific blockers and antagonists.

The α subunits of the Kv1 family are mammalian homologues of the Shaker subunits originally described in *Drosophila* (see Baumann et al. 1988; Schwarz et al. 1988; Tempel et al. 1987, 1988). Multiple members of the Kv1 subfamily (1.1 through 1.6) have been found in the central nervous system (Coetzee et al. 1999). The α subunits of Kv1 are known to form heteromultimers (Christie et al. 1990; Isacoff et al. 1990; Ruppersberg et al. 1990; Wang et al. 1993). Studies have demonstrated that in the nervous system Kv1.1 can combine with Kv1.2, Kv1.2 and Kv1.6, or Kv1.4 (Scott et al. 1994; Wang et al. 1999). However, homomers of Kv1.1 have not been detected in the nervous system (Scott et al. 1994; Shamotienko et al. 1997; Wang et al. 1999). In contrast, when expressed in heterologous cell systems, Kv1.1 gave rise to a rapidly activating, sustained outward current (Bosma et al. 1993; Robertson and Owen, 1993; Stuhmer et al. 1988). In the nervous system, Kv1.1 appears to be widely distributed; it is highly expressed in the juxtaparanodal regions of myelinated axons. However, the channel is
also expressed in unmyelinated axons, cell somas, axon terminals, and in some dendrites (Wang et al. 1993, 1994). Disruption of the Kcna1 gene (the gene locus for Kv1.1) has significant behavioral consequences. These Kv1.1 knock-out mice displayed frequent spontaneous seizures which begin to appear about three weeks postnatally with about half of the mice dying suddenly between weeks three and five. Those mice that lived to adulthood continued to display spontaneous seizures (Rho et al. 1999; Smart et al. 1998). Recordings from neurons in Kcna1 null animals, demonstrated that less current was required to produce AP firing and that these Kv1.1-null neurons typically fired many APs compared to the single AP observed in the wildtype mice (Brew et al. 2003; Smart et al. 1998). These results indicate that Kv1.1 plays a critical role in setting both the point at which a neuron generates an AP and for a given stimulus whether the cell fires single or multiple APs.

Early studies in sensory neurons isolated from either DRG or nodose ganglia showed that dendrotoxin inhibited a slowly inactivating outward I\textsubscript{K} (Penner et al. 1986; Stansfeld et al. 1986, 1987, 1988). Current clamp recordings demonstrated that in rat sensory neurons isolated from the nodose ganglia exposure to α-dendrotoxin (α-DTX), an antagonist for the α subunits of Kv1.1, Kv1.2 and Kv1.6 (Grissmer et al 1994; Grupe et al. 1990; Harvey and Karlsson 1980) lowered the firing threshold of the AP and increased the frequency of firing (Stansfeld et al. 1986; Glazebrook et al. 2002). Similarly, in neurons isolated from trigeminal ganglia, α-DTX increased the number of APs evoked by a step of current without changing the resting membrane potential (Yoshida and Matsumoto, 2005). In addition, the Kv1 family appears to play an important role in modulating neuronal activity under pathological conditions. In rat sensory neurons of the lumber DRG, hypoxia increased the excitability through a suppression of α-DTX-sensitive potassium currents (Gruss et al. 2006). Taken together, these findings suggest that the Kv1.1, Kv1.2, and Kv1.6 subtypes likely play an important role in regulating cell excitability due to the suppression of α-DTX-sensitive potassium currents under normal as well
as pathological conditions. However, due to the expression of multiple channel subtypes as well as their capacity to form heteromultimers, it is not clear which specific subtypes function in modulating particular aspects of excitability in sensory neurons.

To address this question, we used the specific blocker of Kv1.1, dendrotoxin-K (DTX-K, IC\textsubscript{50} \(\sim\) 2 nM, Owen et al. 1997; Robertson et al. 1996) and siRNA targeted to Kv1.1 to reduce specifically the expression of this channel and thereby determine the role of Kv1.1 in modulating the excitability of small diameter capsaicin-sensitive sensory neurons. In this report, we demonstrate that exposure to DTX-K augmented the number of APs evoked by a ramp of current and that this increased excitability was associated with changes in specific parameters of the AP whereas other properties were unaffected. Reduction of Kv1.1 protein with siRNA produced changes that were consistent with the acute effects of DTX-K. These results suggest that Kv1.1 plays a significant role in regulating the firing properties of sensory neurons.

**METHODS**

*Isolation and maintenance of adult rat sensory neurons*

Sensory neurons were isolated from young adult rats using procedures developed by Lindsay (1998) with slight modifications. Briefly, male Sprague-Dawley rats (100-150 g) were killed by placing them in a chamber filled with CO\textsubscript{2}. The dorsal root ganglia (DRG) were collected in a culture dish filled with sterilized Puck’s solution. The ganglia were transferred to a conical tube with F-12 media containing papain (20 U/ml) and incubated for 15 min at 37\(^\circ\) C, followed by incubation in 1 mg/ml collagenase IA and 2.5 mg/ml dispase for 10 min. The suspension was centrifuged (~2000 x g) for 30 s before the enzyme-containing supernatant was removed. The pellet was resuspended in F-12 media supplemented with 250 ng/ml nerve growth factor and mechanically dissociated with fire-polished pipettes until all obvious chunks of tissues were
gone. Isolated cells were maintained at 37° C and 3% CO₂ and used within 24 hrs and 48 hrs for electrophysiological/RT-PCR studies and the confocal immunohistochemical studies, respectively. In results described for all other experiments, e.g., the DTX-K and siRNA experiments, the isolated neurons were maintained in culture for a total of 7 days, the media was changed every two days. All procedures have been approved by the Animal Use and Care Committee of the Indiana University School of Medicine.

**Electrophysiology**

Recordings were made using the whole-cell patch-clamp technique as previously described (Hamill et al. 1981; Zhang et al. 2006). Briefly, a cover slip with the sensory neurons was placed in a recording chamber where neurons were bathed in normal Ringers solution of the following composition (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES and 10 glucose, pH at 7.4 with NaOH. Whole-cell currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The whole-cell recording configuration was established in normal Ringers solution. Both capacitance and series resistance compensation (typically 80%) were used. Leak subtraction was not used for the measurement of the potassium current (I_K) so that any effects of these agents on the holding current could be determined. To assess excitability in the current clamp experiments, neurons were held at their resting potentials (range -50 to -65 mV) and a depolarizing ramp of current (0.9 s in duration) was applied. The amplitude of the ramp was adjusted to produce two to four APs (sampling rate 1 KHz) under control conditions then the same ramp was used throughout the recording period for each individual neuron.

To isolate I_K, neurons were superfused with a Ringers solution wherein NaCl was substituted with equimolar N-methyl-glucamine chloride (NMG-Cl, 140 mM); pH was adjusted to 7.4 with
KOH. Recording pipettes typically had resistances of 2-4 MΩ when filled with the following solution (in mM): 140 KCl, 5 MgCl₂, 4 ATP, 0.3 GTP, 2.5 CaCl₂, 5 EGTA (calculated free Ca²⁺ concentration of ~100 nM, MaxChelator) and 10 HEPES; pH was adjusted to 7.3 with KOH. This pipette solution was used in the current clamp recordings as well. The membrane was held at -60 mV; this value was chosen so that current measurements could be ascertained at a voltage that reflected the normal resting potential in these sensory neurons. Activation of Iᵦ was determined by voltage steps of 100 ms, which were applied at 5 s intervals in +10 mV increments from -80 to +40 mV. At the end of these steps, the voltage was set to -40 mV for 20 ms, after which it returned to the holding potential of -60 mV. The currents were filtered at 5 KHz and sampled at 1 KHz using pClamp 8.0 (Molecular Devices). After obtaining the control response, the bath solution was changed to the appropriate Ringers solution and cells were superfused continuously for the appropriate times. In a separate series of time control experiments, the maximum current amplitudes during the voltage step for Iᵦ did not vary significantly over a 20 min time period indicating that there was little run-down of this current over this time. At the end of each recording, the neuron was exposed to 100 nM capsaicin. This neurotoxin was used to distinguish capsaicin-sensitive sensory neurons as these neurons are believed to transmit nociceptive information (Holzer, 1991). However, the correlation between capsaicin sensitivity and that a neuron is a nociceptor is not absolute. Some nociceptive neurons are insensitive to capsaicin and some capsaicin-sensitive neurons are not nociceptors (see Petruska et al. 2000). Therefore, this agent was used to define a population of small diameter sensory neurons that could serve a nociceptive function. The results reported below were obtained from capsaicin-sensitive neurons only. All experiments were performed at room temperature (~22°C).

Data analysis
Data are presented as the mean ± standard error of the mean (S.E.M.). The AP firing threshold was determined by differentiating the voltage trace (dV/dt) evoked by the ramp in current clamp recordings. The voltage and time at which the first AP was fired were taken as the point that exceeded the baseline value of dV/dt by >20-fold. The baseline value of dV/dt was determined by averaging the points over 100 ms that began with the onset of the current ramp (65-165 ms). The rheobase was measured as the amount of ramp current at the firing threshold. To evoke a single AP (sampled at 10 KHz), steps of current in 400 pA increments and 25 ms in duration were used. The duration of the AP was measured at half the total amplitude of the AP. Statistical differences between the control recordings and those obtained under various treatment conditions were determined by using either a t-test, paired t-test, analysis of variance (ANOVA), or repeated measures ANOVA (RM ANOVA). When a significant difference was obtained with an ANOVA, post hoc analyses were performed using a Tukey test. Values of p<0.05 were judged to be statistically significant.

**RT-PCR**

The presence of gene transcripts for Kv1.1 was detected after electrophysiological recording using techniques described by Song et al. (1998) with modification. Briefly, I_K was recorded from a small diameter sensory neuron; the cell was aspirated into another sterilized micropipette containing 5 µl DEPC water. The contents of the micropipette were forced into a microtube and the RNA was reverse transcribed using the SuperScript first strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was stored at -20° C prior to the PCR detection and amplification of Kv1.1 using the forward primer (bases 1573-1595): CCGCCGCAGCTCCTCTACTATCA and the reverse primer (bases 1781-1758): CAAGGGTTTTGTTTGGGGGCTTTT using the Platinum PCR Supermix (Invitrogen). These PCR reactions ran for 45 cycles (94° C for 1 min, 51° C for 1 min, 72° C for 2 min). The
PCR product was sequenced using an ABI Prism 3100 genetic analyzer at facilities in the Department of Biochemistry and Molecular Biology, Indiana University School of Medicine.

**Immunohistochemistry**

Fluorescence labeling of Kv1.1 was performed in isolated sensory neurons. Isolated neurons were grown on glass bottom microwell dishes (MatTek Corp, Ashland, MA) for 48 hrs and were washed with PBS without Ca\(^{2+}\) or Mg\(^{2+}\) (PBS-CM, 0.1 M, pH 7.4). The cells were fixed with 4% paraformaldehyde for 10-15 min and permeabilized with 0.025% saponin for 2 min at room temperature. After extensive washing with PBS-CM, cells were blocked with 2% bovine serum albumin and 0.025% saponin and fish skin gelatin in PBS-glycine (10 mM) for 5 min at room temperature. To detect Kv1.1, the cells were incubated with the primary antibody for Kv1.1 (1:100) overnight at 4\(^\circ\) C. After washing in PBS-CM (3 times, 5 min each), cells were incubated in darkness for 1 hr at room temperature with a secondary anti-rabbit Cy5-conjugated antibody (1:200, Jackson Immunoresearch, West Grove, PA) and washed in PBS-CM (3 times, 5 min each). Fixed cells were immersed in SlowFade antifade reagent (Molecular Probes, Eugene, OR). Immunofluorescence was observed using confocal microscopy. Images were collected using a Zeiss LSM510 microscope equipped with a Zeiss X63 water immersion lens. Optimal images were obtained by averaging two scans. The excitation and emission wavelengths for Cy5 were 650 and 680 nm, respectively.

**siRNA**

An siRNA sequence corresponding to nucleotide positions 599-619 (AAATTTTACGAGTTGGGCGAG) of rat Kv1.1 mRNA (NM_173095) was selected according to the software provided by the Dharmacon siDESIGN website (dharmacon.ordersassistance@thermofisher.com). siRNA was synthesized by Dharmacon. To reduce expression, the siRNA treatment protocol and sequence for siRNA targeted to
apurinic/apyrimidinic endonuclease (Ape1) were used as previously described by Vasko et al. (2005). Briefly, isolated sensory neurons were maintained for 2 days in normal media with 250 ng/ml NGF. Normal media was replaced with F-12 media lacking antibiotics and bathed the cells for ~6 hrs. The cells were rinsed once with Optimem media and incubated at 37º C for ~30 min. The Neuroporter-siRNA complex (100 nM) was added on day 3 in culture wherein the neurons were exposed to the siRNA or Neuroporter alone for 48 hrs at 37º C. After 2 days (day 5 in culture), the Neuroporter -/+ siRNA was washed out and the normal media containing antibiotics and NGF was then added to the neurons and allowed to incubate for another 2 days before electrophysiological recordings or Western blots were performed.

**Western blot**

Isolated sensory neurons either in the absence or presence of siRNA for Kv1.1 were sonicated in fresh TNN-SDS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Nonidet P-40, 50 mM NaF, 20 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.1% SDS, and 2 mM phenylmethylsulfonyl fluoride). The cell lysates were prepared in TNN-SDS buffer at 4º C for 30 min followed by centrifugation (10,000 x g for 10 min). Protein concentration was measured using the Bradford method. Equivalent amounts of protein (30 µg) were loaded and separated on a NuPAGETM 4-12% Bis-Tris Gel (Invitrogen) before transfer to a PVDF membrane (Invitrogen) for western blot analysis. After serial incubation with specific antibodies, immunoreactive bands on the membrane were developed by ECL kit (Amersham Biosciences, Piscataway, NJ) and visualized by exposure to Kodak LS X-OMAT film. The density of the bands was measured by Quantity One software (Bio-Rad Life Science Research, Hercules, CA).

**Chemicals**

Dendrotoxin-K and the primary antibody to Kv1.1 used in the immunohistochemical studies were obtained from Alomone Labs (Jerusalem, Israel). The primary antibody to Kv1.1 used in
the western blots was obtained from Sigma Chemical Corp. (St Louis, MO). The primary antibody to Kv1.2 used in the western blots was obtained from NeuroMab (Davis, CA). Tissue culture supplies were purchased from Invitrogen (Carlsbad, CA). All other chemicals were obtained from Sigma Chemical Corp. (St Louis, MO). Capsaicin was dissolved in 1-methyl-2-pyrrolidinone to obtain concentrated stock solutions. The stock solutions were then diluted with Ringers to yield the appropriate concentration. We have demonstrated previously that the vehicle, 1-methyl-2-pyrrolidinone, has no effect on AP firing or the activation of I\(_\text{K}\) (Zhang et al. 2002).

RESULTS

*Small diameter sensory neurons express Kv1.1*

To determine whether a small diameter sensory neuron expressed the mRNA for Kv1.1, single cell RT-PCR was used in combination with whole-cell patch-clamp recording. The outward current obtained from a representative neuron is shown in Fig. 1 (top panel). These currents exhibited the rapid activation and little time-dependent inactivation that is characteristic of delayed rectifier potassium currents (I\(_\text{K}\)). These currents are similar to those observed when the cloned Kv1.1 was expressed in a heterologous expression system (see Fig. 1, Bosma et al. 1993). After recording the outward current, the neuron was aspirated into a pipette from which the single-cell RT-PCR was performed. As shown in the bottom panel of Fig. 1, lane A demonstrates that the PCR product obtained from the cDNA from five small diameter sensory neurons was of the correct product size (208 bp). In addition, the PCR product was sequenced and found to match exactly the targeted bases. The PCR product for Kv1.1 obtained from the single neuron exhibiting the delayed rectifier-like current in the top panel (lane B) was at the same base pair size, although less intense, as that obtained from the five neurons (lane A). Lane C indicates that in the absence of cDNA, no PCR product was obtained. In cDNA
obtained from rat brain, a PCR product of 208 bp was also detected (data not shown). These results indicate that small diameter sensory neurons express the mRNA for Kv1.1. To further support the RT-PCR measurements, immunohistochemistry was used to determine whether the Kv1.1 protein was expressed in the neurons. Incubation with the Kv1.1 antibody stained small, medium, and large diameter neurons with varying degrees of fluorescence intensity although not every neuron expressed Kv1.1 (see panels in Fig. 2A-E). In the absence of primary antibody, no reaction was observed with the secondary antibody alone (see Fig. 2F). Also, expression of Kv1.1 was detected in western blots obtained for the control conditions in the siRNA studies (see top right panel, Fig. 4). Taken together, these results demonstrate that sensory neurons express the potassium channel Kv1.1 and are consistent with previous reports (see Discussion).

DTX-K increases the excitability of small diameter capsaicin-sensitive sensory neurons

We sought to establish whether the specific blocker of Kv1.1, DTX-K, could alter the excitability of small diameter capsaicin-sensory neurons. The current clamp configuration was used to examine the effects of DTX-K on the capacity of sensory neurons to fire APs when stimulated with a ramp of depolarizing current. As shown for a representative neuron (Fig. 3A), a 5 min exposure to 10 nM DTX-K increased the number of APs evoked by the current ramp from a control value of 2 APs to 10 APs. The results for the current clamp experiments obtained from a total of eleven small diameter sensory neurons are summarized in Fig. 3B. Exposure to DTX-K significantly increased the number of APs from a control value of 2.5 ± 0.3 to 7.7 ± 1.5 APs (paired t-test) after a 5 min application. The increase in AP firing produced by DTX-K was accompanied by a significant hyperpolarizing shift of ~4 mV in the firing threshold and a 50% reduction in the rheobase (see Table 1, n=11, paired t-test). However, neither the resting membrane potential nor the duration of the AP was altered by DTX-K (see Table 1). Most of these untreated neurons (7 of the 11) exhibited an inflection (a hump) on the falling phase of the
AP (assessed by dV/dt). Rat sensory neurons exhibiting this inflexion were associated with slower conduction velocities (C and Aβ fibers; Harper and Lawson, 1985) and are presumed to be characteristic of identified nociceptive neurons in both cat and rat (Koerber et al. 1988; Ritter and Mendell, 1992). Thus, these DTX-K-induced changes in the number of APs and the firing threshold are indicative of the important role that Kv1.1 plays in regulating each respective parameter and its contribution to the total excitability of these neurons.

siRNA treatment reduces the expression of Kv1.1

To establish the functional contribution of Kv1.1 to neuronal excitability, expression of this channel protein was reduced using siRNA targeted to the α-subunit of Kv1.1 (amino acids 599-619, from the origin). These results are summarized in Fig. 4. The top panels show representative western blots obtained for dishes of isolated sensory neurons (~5000 cells) undergoing three different experimental treatments. The right lane represents the untreated control condition wherein Kv1.1 was detected at the expected size of ~80 KDa in isolated sensory neurons that were in culture for seven days; the same length of time as for the siRNA treatment. The middle lane represents the detection of Kv1.1 in neurons that were exposed to siRNA targeted to apurinic/apyrimidinic endonuclease (Ape1), which is a DNA repair enzyme. This siRNA has been used in Dr. Michael Vasko’s laboratory in other studies where it effectively reduced the expression of Ape1 (Vasko et al. 2005). Here, it serves as a negative control. The left lane shows that after treatment with siRNA targeted to Kv1.1, the expression was reduced compared to either the control or Ape1 siRNA-treated neurons. The densitometries are summarized in the bottom panel wherein Kv1.1 siRNA significantly reduced the expression of Kv1.1 by 54 ± 9% (n=6 separate tissue harvests) whereas the Ape1 siRNA had no effect (n=2 separate tissue harvests for each condition). In addition, to examine the specificity of the siRNA targeted to Kv1.1, a separate series of experiments determined the expression levels of Kv1.2, a potassium channel α subunit known to associate with Kv1.1 (see Introduction), after a 48 hr
exposure to siRNA targeted to Kv1.1. Analysis of the densitometry showed that the expression level of Kv1.2 (when normalized to the density of actin in each respective lane) was unchanged after siRNA treatment when compared to untreated neurons undergoing the same procedure (control Kv1.2/actin 1.0 vs. siRNA-treated 0.99 ± 0.12, n=3, data not shown). However, when the same gels were probed for Kv1.1, treatment with siRNA targeted to Kv1.1 produced a significant decrease in the expression of Kv1.1 (control Kv1.1/actin 1.0 vs. siRNA-treated 0.59 ± 0.04, n=3, data not shown). This reduction of 41% was not different than the 54% decrease (p=0.48, t-test) reported above for a separate series of experiments. The lack of effect of siRNA targeted to Ape1 on Kv1.1 expression and the inability of siRNA targeted to Kv1.1 to affect expression of Kv1.2 suggest that siRNA can be used to effectively reduce the expression levels of targeted channels.

**DTX-K suppressed outward potassium currents in untreated but not in siRNA-treated neurons**

To confirm that siRNA targeted to Kv1.1 reduced the functional expression of this channel protein, the inhibitory effects of DTX-K, the antagonist of Kv1.1, on $I_K$ in untreated control or in neurons exposed to siRNA was examined. Under control conditions, in a representative untreated neuron, the amplitude of the outward $I_K$ was 4.62 nA measured at +40 mV (Fig. 5A, top left panel). After a 5 min exposure to 10 nM DTX-K, the amplitude of $I_K$ decreased to 2.52 nA (top middle of panel A). These results are summarized in the current-voltage relation shown in Fig. 5A (right panel). Under control conditions, the outward $I_K$ began to activate at approximately -30 mV. In the untreated neurons, 10 nM DTX-K significantly inhibited the $I_K$ obtained at +40 mV by 33 ± 6% (control 5.02 ± 1.01 nA vs. 3.24 ± 1.08 nA, n=6, paired t-test) after a 5 min exposure (Fig. 5A, right panel). $I_K$ was suppressed by 40 ± 9% after 10 min in these same neurons (data not shown). These results indicate that the maximum inhibition was attained after ~5 min and that there was not a longer time-dependent component to the
inhibition produced by DTX-K. In the left panel of Fig. 5B, the $I_K$ sensitive to DTX-K is shown (same neuron in panel A) and demonstrates that this current exhibits little time-dependent inactivation. The current-voltage relation for the DTX-K sensitive $I_K$ is summarized in the middle panel of B. The DTX-K sensitive $I_K$ begins to activate at about $-10 \text{ mV}$, which is $\sim 20 \text{ mV}$ more depolarized than observed for the total $I_K$ under control conditions. The right panel in B illustrates a comparison of the normalized current-voltage relations for the control and the DTX-K sensitive $I_K$s. In contrast, in a representative neuron after treatment with siRNA targeted to Kv1.1 (left and middle panels of Fig. 5C), exposure to DTX-K did not alter $I_K$ (control 3.67 nA measured at $+40 \text{ mV}$ compared to 3.98 nA after DTX-K). The current-voltage relations for these two conditions are summarized in the right panel of Fig. 5C. Exposure to DTX-K did not alter the amplitude of $I_K$ measured at $+40 \text{ mV}$ (control $3.51 \pm 0.49 \text{ nA}$ vs. 5 min DTX-K $3.48 \pm 0.42 \text{ nA}$, n=6, paired t-test). Since all of these recordings were obtained from only small diameter sensory neurons, the reduction in the total $I_K$ measured at $+40 \text{ mV}$ from siRNA-treated neurons suggests that the total current density was decreased after siRNA treatment, which is consistent with the loss of conducting Kv1.1. Because of the specificity of DTX-K for Kv1.1, these results clearly demonstrate that siRNA treatment was capable of reducing the expression of Kv1.1 to the point where DTX-K inhibition of $I_K$ at a concentration five times greater than the IC$_{50}$ was no longer detected.

The capacity of DTX-K to augment excitability is prevented by siRNA treatment

Treatment with siRNA targeted to Kv1.1 lowers the expression of Kv1.1, but functionally what are the consequences of this reduction? To examine this question, the parameters of excitability described above were examined in the absence and presence of DTX-K in Kv1.1 siRNA-treated neurons. In a representative Kv1.1 siRNA-treated neuron, the ramp of depolarizing current evoked 3 APs under control conditions (see Fig. 6A left panel). After a 5
min exposure to 10 nM DTX-K (right panel), this neuron fired only 2 APs, indicating that the excitation was not affected by DTX-K. In a total of 15 sensory neurons, a 5 min exposure to 10 nM DTX-K did not significantly increase the number of APs after treatment with siRNA targeted to Kv1.1 (control 3.1 ± 0.2 vs. 3.5 ± 0.6, p=0.52, paired t-test). However, in three of the fifteen neurons, DTX-K caused a two-fold increase in the number of APs, suggesting that these particular neurons did not take-up the siRNA as the DTX-K-induced increase in the number of APs was quite similar to that observed in the untreated neurons. Based on this, these three neurons were excluded from further analysis. (The values for the number of APs between the control and treated groups for the fifteen neurons was not different from the twelve neurons, p=0.31 ANOVA). The results obtained from twelve small diameter capsaicin-sensory neurons are summarized in Fig. 6B. In the Kv1.1 siRNA-treated neurons, a 5 min exposure to DTX-K did not alter the number of evoked APs (control 3.0 ± 0.2 vs. 2.4 ± 0.3 after 5 min, paired t-test). These results indicate that DTX-K was incapable of affecting the excitation after treatment with siRNA targeted to Kv1.1. As with the number of APs, exposure to DTX-K did not change the properties of neurons including the firing threshold, or the rheobase (see Table 1). Similar to the untreated neurons, DTX-K did not affect the resting membrane potential or the duration of the AP (11 of 12 neurons exhibited an AP “hump”) after Kv1.1 siRNA treatment (Table 1, n=12, paired t-test). Consistent with the idea that siRNA reduced the expression of Kv1.1, in the siRNA-treated neurons, the average value of the firing threshold was close to that measured after a 5 min exposure to DTX-K in the untreated neurons (-16.8 ± 4.0 for siRNA neurons vs. -15.1 ± 3.2 mV for untreated). However, the average value of the rheobase determined in the siRNA-treated neurons was not different from the value obtained for the untreated neurons under control conditions (before DTX-K). These results suggest that after treatment with siRNA targeted to Kv1.1, other potassium channels may assume an important role in setting the rheobase after the loss of Kv1.1. It is also possible that the siRNA targeted to Kv1.1 affected an “off-target” site which influences the rheobase that we are unaware of presently. In sensory
neurons that were exposed to siRNA targeted to Ape1, treatment with DTX-K produced effects that were similar to those observed for untreated neurons. For example, a 5 min exposure to 10 nM DTX-K significantly increased the number of evoked APs (control value 2.3 ± 0.5 vs. after DTX-K 7.8 ± 1.5 APs, n=4, paired t-test). In these Ape1 siRNA-treated neurons, DTX-K had no effect on the resting membrane potential (control -60.0 ± 3.4 vs. after DTX-K -59.0 ± 3.4 mV, n=4, paired t-test) or the AP duration (control 5.7 ± 0.8 vs. after DTX-K 5.7 ± 1.3 ms, n=4). Similar results were obtained for those neurons exposed to the transfecting agent, Neuroporter, alone. For example, DTX-K significantly increased the number of APs from a control value of 2.7 ± 0.9 to 6.0 ± 1.2 and decreased the firing threshold by about 10 mV without altering the resting membrane potential (data not shown, n=3, paired t-test). These observations indicate that Kv1.1 is important in establishing the firing threshold/rheobase of sensory neurons and may be an important target for further modulation of the excitability in sensory neurons.

Discussion

Our results demonstrate that sensory neurons of the DRG express the potassium channel Kv1.1 and that this channel plays an important role in setting the level of excitability. This channel appears to be expressed in neurons of all sizes. Although our observations are not quantitative, they are consistent with previous reports demonstrating that Kv1.1 was present at moderate to high levels (based on an optical density-gray scale calibration) in both small (<30 µm) and medium to large (>30 µm) diameter sensory neurons of the L4/5 DRG of the rat (Ishikawa et al. 1999) and in most neurons (89%) of the nodose ganglia (Glazebrook et al. 2002). These findings are in contrast to those showing that Kv1.1 was detected primarily in large diameter neurons of the L4-6 DRG (Rasband et al. 2001). The reasons for these differences are not presently clear. In addition, others have reported the expression of the mRNA for Kv1.1 in the L4/5 DRGs using either RT-PCR (Kim et al. 2002) or RNase protection assays (Yang et al. 2004). Further studies using the combined approach of electrophysiological recordings and
single-cell RT-PCR would prove useful in clarifying the distribution of Kv1.1 in the neurons of the DRG.

Early studies indicated that dendrotoxin inhibited a slowly inactivating outward $I_K$ in sensory neurons isolated from either the DRG or nodose ganglia (Penner et al. 1986; Stansfeld et al. 1986, 1987, 1988). More recent work with $\alpha$-DTX, which is specific for Kv1.1, Kv1.2, and Kv1.6, demonstrated that in nodose ganglia (Glazebrook et al. 2002) or DRG (Yang et al. 2004) $I_K$ was reduced by $\sim20\%$ as measured at $+15$ and $+20$ mV, respectively, whereas in trigeminal ganglia, $I_K$ was reduced by only $\sim12\%$ (Yoshida and Matsumoto 2005). Studies which have used the Kv1.1-selective DTX-K showed that $I_K$ was reduced by $\sim34\%$ at $+20$ mV in sensory neurons isolated from the mouse DRG (Beekwilder et al. 2003) or the rat nodose ganglia (Glazebrook et al. 2002). These observations are similar to the extent of inhibition of $I_K$ by DTX-K in capsaicin-sensitive small diameter sensory neurons that we described above ($\sim33\%$ inhibition at $+40$ mV).

In contrast, recordings from auditory neurons of the medial nucleus of the trapezoid body (MNTB) demonstrated that DTX-I (an $\alpha$-DTX analog isolated from the black mamba snake) more effectively inhibited a low threshold $I_K$ than did TEA over the voltage range of -50 to -10 mV whereas a high threshold $I_K$ exhibited greater sensitivity to TEA compared to DTX-I (Brew and Forsythe 1995; Brew et al. 2003; Dodson et al. 2002). It is difficult to directly assess the contribution of either Kv1.1 or Kv1.2 to the current between -50 and -10 mV because there is only a 10-fold difference in the IC$_{50}$s of DTX-I for Kv1.1 (3 nM) and Kv1.2 (0.4 nM) (Hopkins 1998). However, DTX-K produced a similar amount of block as did DTX-I suggesting that the low threshold $I_K$ was conducted by Kv1.1/Kv1.2 heteromultimers (Dodson et al. 2002). The similar efficacy of DTX-K and DTX-I in neurons of the MNTB is in contrast to the observations in sensory neurons of the nodose ganglia where DTX-K produced an additional block of $\sim60\%$ of the current remaining after exposure to $\alpha$-DTX (Glazebrook et al. 2002). These results indicate
that the current sensitive to α-DTX is not conducted entirely by Kv1.1/Kv1.2 heteromultimers. Thus, such observations suggest that either the complement or oligomeric complexes of potassium channels and/or the contributions of DTX-sensitive potassium channels to excitability in primary afferent neurons may be different than those described for auditory neurons of the MNTB (e.g. Scott et al. 1994).

Neurons express a multitude of potassium channels, which raises the question as to the contribution of specific channels, such as Kv1.1, to the regulation of AP firing or excitability in general. In our studies, inhibition of Kv1.1 produced about a two-fold increase in the number of APs evoked by a ramp of depolarizing current. Associated with the increased AP firing were reductions in both the firing threshold and the rheobase. These are all factors that indicate that Kv1.1 plays an important role in controlling the excitability of these sensory neurons. Although DTX-K augmented AP firing, neither the resting membrane potential nor the AP duration were altered suggesting that Kv1.1 makes little contribution to these two particular parameters. It has been reported that dendrotoxins produced similar enhancements in excitability without changes in resting membrane potential nor AP duration in sensory neurons isolated from the nodose and trigeminal ganglia (Glazebrook et al. 2002; Stansfeld et al. 1986; Yoshida and Matsumoto 2005) as well as in neurons of the MNTB (Brew and Forsythe 1995; Brew et al. 2003). Furthermore, the notion of Kv1.1 playing a key role in regulating excitability is supported by recordings obtained from mice wherein the gene for Kv1.1 has been deleted. In recordings from pyramidal neurons of the hippocampal CA3 region, the threshold for AP firing resulting from either mossy fiber or antidromic stimulation was reduced and rather than evoking a single AP as in the wildtype mice, a train of APs was observed in the Kcnai-null mice (Lopantsev et al. 2003; Smart et al. 1998). The resting membrane potentials and the AP durations were not different in the knock-out and the wildtype mice.
We used siRNA to specifically reduce the expression level of this potassium channel. This approach has the advantage over gene deletion in that neurons develop with their normal complement of channels and the target in question is reduced only transiently. Treatment with siRNA targeted to Kv1.1 reduced the protein expression by about 60% and completely removed the inhibition of $I_K$ produced by DTX-K. It is possible that the remaining 40% of Kv1.1 after siRNA treatment comes from neurons which did not take up the siRNA and/or the detected channel protein was that remaining in intracellular compartments as part of their trafficking (Manganas and Trimmer, 2000). In terms of the excitability, after siRNA treatment DTX-K no longer increased the number of APs evoked by the ramp as observed in the untreated, the Neuroporter controls, or the Ape1 siRNA-treated neurons. Thus, it seems likely that the Kv1.1-like immunoreactivity detected by the western blots after siRNA treatment does not represent functional protein. After treatment with Kv1.1 siRNA, several parameters exhibited values that were similar to those determined after exposure to DTX-K in untreated neurons. For example, the firing threshold after siRNA treatment reflected those values measured after DTX-K, indicating that the transient inhibition produced by DTX-K had similar actions as that caused by reducing the expression level of Kv1.1. In untreated neurons, the rheobase was significantly reduced after DTX-K, however, in siRNA-treated neurons the rheobase was similar to that determined for the control value rather than that after DTX-K. The reasons for this are presently unclear. One possibility may involve the heteromeric interactions of Kv1.1 with other potassium channel subunits, such as Kv1.2 or Kv1.6, which may be involved in regulating the rheobase. This notion regarding the contribution of potassium channel heteromultimers is quite complex and clearly requires more detailed investigation.

siRNA has proven to be effective in blocking the actions of other potassium channels (see Gurney and Hunter, 2005). In CHO cells, both the expression and current conducted by Kv4.3 was reduced by approximately 75% after a 48 hr treatment with siRNA (Cotella et al. 2005). In
HEK 293 cells expressing the two-pore potassium channel TASK-2, siRNA reduced the expression by about 64% and the current measured at pH 7.4 by 40%. Treatment with the scrambled siRNA had no effect. When this siRNA was used in isolated pulmonary artery myocytes, the depolarization produced by a TASK-2 selective inhibitor was reduced significantly compared to the untreated myocytes (Gonczi et al. 2006). These results suggest that siRNA can effectively reduce the expression of ion channels so that their functional aspects can be established in native tissues.

In summary, our results demonstrate that suppression of Kv1.1 by exposure to the selective blocker DTX-K produced an increase in AP firing that was associated with decreases in the firing threshold and the rheobase. Also, siRNA targeted to Kv1.1 reduced the expression of this potassium channel in which the actions of DTX-K to inhibit $I_K$ and enhance AP firing were prevented. Thus, dendrotoxin-sensitive potassium channels play an important role in limiting the excitability of sensory neurons. Treatments with siRNA should prove to be effective in establishing the physiological roles of ion channels in excitability where selective antagonists are not yet available.

Acknowledgement

We would like to thank Drs. Michael Vasko, Chun Lu Guo, and Mr. Eric Thompson for their advice regarding siRNA techniques.

Grants

This investigation was conducted in a facility constructed with support from Research Facilities Improvement Program Grant Number C06 RR015481-01 from the National Center for Research Resources, NIH. This work was supported, in part, by an award from the Ralph W. and Grace M. Showalter Research Trust Fund.
Disclosure

The authors have no conflicts to declare.

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Figure Legends

Figure 1. Using single-cell RT-PCR, the mRNA for Kv1.1 was detected in a small diameter sensory neuron that exhibited outward I_K. The top panel shows a representative I_K evoked from a small diameter sensory neuron. The voltage steps are in +20 mV increments from -80 to +60 mV. The bottom panel illustrates that the mRNA for Kv1.1 was detected in a sample from 5 small diameter neurons (lane A) and in the single neuron from which the recording in the top panel was obtained (lane B). No PCR products were detected in the no template control (lane C). The base pair ladder is shown on the left.

Figure 2. Sensory neurons stain positive for Kv1.1. These panels (A-E) show that the protein for Kv1.1 was expressed in small, medium, and large diameter sensory neurons. The top panel represents the fluorescence image whereas the bottom panel represents the bright-field image. These images were obtained from the same harvest of sensory neurons and were obtained at the same confocal settings. Panel A shows that a medium diameter neuron is positive for Kv1.1 whereas the two smaller neurons are negative (indicated by the arrows). Panel B shows a small diameter neuron that is negative for Kv1.1. Panels C and E show two small diameter neurons wherein one is positive and one is negative (arrows) for Kv1.1. Panel D shows a large diameter neuron positive for Kv1.1 and a medium diameter that is negative (arrow). Panel F shows a representative image obtained in the absence of primary antibody indicating the specificity of this reaction. The scale bar in A represents 30 µm and applies to all panels.

Figure 3. DTX-K, a specific blocker of Kv1.1, increased the number of APs in untreated control sensory neurons. Panel A illustrates a current clamp recording from a representative neuron wherein the ramp of depolarizing current elicited 2 APs under control conditions (left panel) and 10 APs after a 5 min exposure to 10 nM DTX-K (right panel). The resting membrane was -60 mV under both treatment conditions. Panel B summarizes the results obtained for eleven
neurons and shows that DTX-K augments the excitability of these neurons. The asterisk indicates a significant difference from the control values (p<0.05, paired t-test).

Figure 4. siRNA treatment lowered the expression of Kv1.1 protein in sensory neurons. The top panel represents an example of Kv1.1 expression detected by western blots for three different experimental treatments. The right lane represents the untreated control condition. The middle lane shows the detection of Kv1.1 in control neurons that were exposed for 48 hrs to siRNA (details in the Methods) targeted to apurinic/apyrimidinic endonuclease (Ape1). The left lane shows that after a 48 hr treatment with siRNA targeted to Kv1.1, the expression of Kv1.1 was reduced. The bottom panel summarizes the normalized blot densities obtained for the Kv1.1- and Ape1-siRNA treatment conditions (n=6 and n=2 tissue harvests, respectively) and the untreated control conditions (n=6 tissue harvests). The blot densities of Kv1.1 for each treatment have been normalized to their respective actin densities (treatment Kv1.1 density/actin) and then divided by the normalized density obtained for the parallel untreated control (control Kv1.1 density/actin). The asterisk indicates a significant difference from the control values (p<0.05, ANOVA).

Figures 5. DTX-K suppressed I_K in untreated but not in siRNA treated neurons. Panel A illustrates the total I_K recorded from a representative untreated neuron (left) where the maximum current amplitude during the voltage step of I_K measured at +40 mV was 4.62 nA. After a 5 min exposure to 10 nM DTX-K (middle), the amplitude of I_K decreased to 2.52 nA. DTX-K produced a significant decrease in I_K between the 0 and 40 mV. The current traces are shown in +20 mV increments for voltage steps between -80 to +40 mV. The lines labelled 0 indicate the zero-current level. The right panel of A summarizes the current-voltage relation before and after a 5 min exposure to DTX-K. Panel B (left) illustrates representative traces for the DTX-K-sensitive
$I_K$ obtained for the neuron in panel A. The middle panel shows the current-voltage relation for the DTX-K-sensitive $I_K$. The right panel of B compares the normalized current-voltage relations for the untreated control $I_K$ and the DTX-K-sensitive $I_K$. Panel C shows total $I_K$ from a representative neuron after treatment with Kv1.1 siRNA (left), the amplitude of $I_K$ was 3.67 nA measured at +40 mV. After a 5 min exposure to 10 nM DTX-K (middle), the amplitude of $I_K$ was 3.98 nA. The current traces are shown in +20 mV increments for voltage steps between -80 to +40 mV. The right panel of C summarizes the current-voltage relation for the Kv1.1 siRNA-treated sensory neurons before and after a 5 min exposure to 10 nM DTX-K.

Figure 6. DTX-K did not alter the number of evoked APs after treatment with Kv1.1 siRNA. Panel A (left) shows a representative recording from a Kv1.1 siRNA-treated neuron under control conditions. The right panel demonstrates that a 5 min exposure to 10 nM DTX-K did not affect the number of APs evoked by the ramp. Panel B summarizes the effects of DTX-K on the number of APs in twelve siRNA-treated sensory neurons.
<table>
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<tr>
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<th>Number of APs</th>
<th>Firing Threshold</th>
<th>RMP</th>
<th>APD</th>
<th>Rheobase</th>
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<td>(mV)</td>
<td>(mV)</td>
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<td>Untreated (n=11)</td>
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<td>5.4 ± 0.8</td>
<td>231.3 ± 49.3*</td>
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<tr>
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<td>8.3 ± 1.4</td>
<td>470.3 ± 134.4</td>
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*p<0.05 paired t-test

RMP: resting membrane potential

APD: action potential duration