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BK_{Ca} currents are enriched in a subpopulation of adult rat cutaneous nociceptive dorsal root ganglion neurons

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Abstract

The biophysical properties and distribution of voltage-dependent, Ca²⁺-modulated K⁺ (BK_{Ca}) currents among subpopulations of acutely dissociated DiI labeled cutaneous sensory neurons from the adult rat were characterized with whole cell patch clamp techniques. BK_{Ca} currents were isolated from total K⁺ current with iberiotoxin, charybdotoxin, or paxilline. There was considerable variability in biophysical properties of BK_{Ca} currents. There was also variability in the distribution of BK_{Ca} current among subpopulations of cutaneous DRG neurons. While present in each of the subpopulations defined by cell body size, IB4 binding or capsaicin sensitivity, BK_{Ca} current was present in vast majority (>90%) of small diameter IB4+ neurons but was present in only a minority of neurons in subpopulations defined by other criteria (i.e., small diameter IB4−). Current clamp analysis indicated that in IB4+ neurons, BK_{Ca} currents contribute to the repolarization of the action potential and adaptation in response to sustained membrane depolarization, while playing little role in the determination of action potential threshold. RT-PCR analysis of mRNA collected from whole DRG revealed the presence of multiple splice variants of the BK_{Ca} channel α -subunit, *rslo* and all 4 of the accessory β subunits, suggesting that heterogeneity in the biophysical and pharmacological properties of BK_{Ca} current in cutaneous neurons, reflects, at least in part, the differential distribution of splice variants and/or β subunits. Because even a small decrease in BK_{Ca} current appears to have a dramatic influence on excitability, modulation of this current may contribute to sensitization of nociceptive afferents observed following tissue injury.

Keywords

primary afferent neuron; sensitization; voltage clamp; nociceptor; *in vitro*

Introduction

Dorsal root ganglia (DRG) contain diverse subpopulations of primary sensory neurons that transduce and encode a variety of stimuli that impact an array of unique tissue types. Whether subpopulations are defined by function (i.e., threshold for activation), adaptation in response to prolonged stimuli (i.e., rapidly or slowly adapting), adequate stimuli (i.e., chemoreceptors), or target of innervation (i.e., cutaneous versus muscle afferents), these subpopulations have distinct electrophysiological properties. These distinct electrophysiological properties reflect the differential distribution of ion channels (Scroggs & Fox, 1992; Gold *et al.*, 1996a).

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Large conductance voltage dependent and Ca^{2+} -modulated K^+ (BK_{Ca}) channels are key regulators of cellular excitability (Sah & Faber, 2002; Stocker *et al.*, 2004). They are characterized by their large single channel conductance, intrinsic voltage dependence, Ca^{2+} modulation, and blockade by charybdotoxin (ChTx) and iberiotoxin (IbTx) and paxilline (Toro *et al.*, 1998; Stocker *et al.*, 2004). Experiments involving selective blockers of BK_{Ca} channels indicate that these channels contribute to membrane repolarization and fast afterhyperpolarization (AHP) following an action potential (Scholz *et al.*, 1998; Pedarzani *et al.*, 2000). Variability in the biophysical properties and pharmacological sensitivity of BK_{Ca} channels has been observed among various excitable tissues as well as within excitable cells (Solaro *et al.*, 1995; Dopico *et al.*, 1999).

While BK_{Ca} currents have been described in sensory neurons (Naruse *et al.*, 1992; Akins & McCleskey, 1993; Scholz *et al.*, 1998; Abdulla & Smith, 2001; Zhang *et al.*, 2003a; Sarantopoulos *et al.*, 2007), there has been no systematic analysis of these currents among subpopulations of afferents defined by target of innervation and putative function. Therefore, the present study was performed to determine whether BK_{Ca} currents are differently distributed among subpopulations of sensory neurons in which putative function was determined on the basis of cell body size, IB4 binding and responsiveness to the algogenic compound capsaicin. Furthermore, because the bulk of data establishing an association between these properties and afferent function *in vivo* has been obtained for cutaneous afferents (Djoughri & Lawson, 2001; 2004; Fang *et al.*, 2005), and because channels may be differentially distributed among subpopulations of afferents based on target of innervation (Oyelese & Kocsis, 1996), this study was restricted to cutaneous afferents identified with the use of retrograde tracers.

Acutely dissociated DRG neurons were studied with perforated and conventional whole cell configurations of patch clamp. BK_{Ca} currents were pharmacologically isolated from total K^+ currents. Our results indicate that there is considerable heterogeneity in the biophysical properties and density of BK_{Ca} currents, even within subpopulations of cutaneous neurons. The current is enriched in a unique subpopulation of putative nociceptive afferents (IB4 binding), where it contributes to action potential repolarization and adaptation in response to prolonged membrane depolarization. Furthermore, the heterogeneity in biophysical and pharmacological properties of BK_{Ca} currents was associated with evidence for the presence of multiple splice variants of the α -subunit, *rslo* (KCNMA1) and all 4 of the accessory β subunits (KCNMB1-4) in DRG neurons. Preliminary data from this study has been published in abstract form (Zhang *et al.*, 2005).

Materials and Methods

Adult (200–250g) male Sprague-Dawley rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) were used for this study. Rats were housed in the University of Maryland Dental School or the University of Pittsburgh animal facility in groups of two or three on a 12:12 light dark schedule. Food and water are available *ad lib*. All experiments were approved by the University of Maryland Dental School and University of Pittsburgh Institution Animal Care and Use Committees and performed in accordance with NIH guidelines as well as guidelines established by the International Association of the Study of Pain for the use of laboratory animals in research. All efforts were employed in order to minimize the number of animals used in this study.

DRG neurons innervating the glabrous skin of the left hindpaw were identified by the administration of the retrograde tracer 1,1'-diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen, Carlsbad CA) into the hindpaw. DiI was dissolved in DMSO (170mg/ml) and diluted 1:10 in 0.9% sterile saline. Under the gaseous anesthesia (isoflurane, 3%) 10 μ l of this solution was injected subcutaneously with a 30-g

injection needle directed into the epithelium. The needle used to inject DiI was inserted through the skin once but the tip of the needle was moved to 4 sites covering an area of ~5 square mm, where 1–1.5 μ l was injected at each site. Labeled neurons were easily identified under epifluorescence illumination.

Cell dissociation

Fourteen to 17 days after injection of DiI into the hindpaw, rats were deeply anesthetized with a subcutaneous injection of rat cocktail (1 ml/kg) consisting of 55 mg/ml ketamine (Fort Dodge Animal Health, Fort Dodge, IA), 11 mg/ml xylazine and 5.5 mg/ml acepromazine (xylazine and acepromazine were from Phoenix Scientific Inc., St. Joseph, MO). Left L₄ and L₅ DRG were removed and desheathed in ice-cold culture media composed of 90% minimal-essential-medium (MEM; Invitrogen), 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biological, Atlanta GA), and 1000 units/ml each of penicillin and streptomycin. Rats were killed by cervical dislocation after DRG harvest. DRG were incubated 45 min at 37°C in 5ml MEM, to which collagenase P (Roche Bioscience, Palo Alto, CA) had been added to a final concentration of 0.125%, and bubbled with carbogen (95% O₂-5% CO₂). DRG were incubated 5 min at 37°C in Ca²⁺- and Mg²⁺-free Hanks balanced salt solution (Invitrogen) containing 0.25% trypsin (Worthington, Bristol, UK), 0.025% EDTA (Sigma, St. Louis MO) and 80 μ g/ml DNase (Sigma). Trypsin activity was inhibited by the addition of MEM-FBS containing 0.125% MgSO₄, and DRG were dissociated by trituration with a fire-polished Pasteur pipette. DRG cells were plated onto glass coverslips, previously coated by a solution of 5 μ g/ml mouse laminin (Invitrogen) and 0.1 mg/ml poly-L-ornithine (Sigma). The cells were incubated in MEM-FBS at 37°C, 3% CO₂, and 90% humidity for 2 h, at which point they were transferred to a HEPES-buffered L-15 media (Invitrogen) containing 10% FBS and 5 mM glucose and stored at room temperature. All neurons were studied between 2 and 8 h after removal from the animal.

Solutions for electrophysiology

Voltage clamp recording were performed using an Axopatch 200B (Molecular Devices, Sunnyvale CA) controlled with pClamp (v 8.2, Molecular Devices). Unless otherwise noted, bath solution contained (in mM): Choline-Cl, 130; KCl, 5; CaCl₂, 2.5; MgCl₂, 0.6; HEPES, 5; and glucose, 10; pH adjusted to 7.4 with Tris-Base, and osmolality adjusted to 320 mOsm with sucrose. Electrode solution for perforated patch clamp recordings contained: (in mM): KCl, 30; K-methanesulfonate (MES), 110; MgCl₂, 1; HEPES, 10; EGTA, 0.1; pH adjusted to 7.2 with Tris-base, and osmolality adjusted to 310 mOsm with sucrose. A similar electrode solution was used for traditional whole cell patch recording, except that in a subpopulation of studies, EGTA was replaced with BAPTA. For current clamp recording, choline-Cl was replaced with NaCl in the bath solution and the electrode solution contained KCl, 30; K-MES, 110; MgCl₂, 1; CaCl₂, 0.1; EGTA, 1; HEPES, 10; ATP-Mg, 2; GTP, 1; pH adjusted to 7.2 with Tris-Base, and osmolality adjusted to 310 mOsm with sucrose. Amphotericin B, used to obtain whole-cell access for perforated patch recording, stock solution was prepared in DMSO (90 mg/ml) then diluted to a final concentration (600 μ g/ml) in electrode solution immediately prior to use. All salts used for electrophysiology were obtained from Sigma-Aldrich.

Experimental protocol

DRG neurons were incubated with FITC (Fluorescein) conjugated *griffonia Simplicifolia* isolectin B4 (IB4, Sigma) for 10 min before recording. The IB4 stained neurons were easily recognized under epifluorescence illumination. With the filter sets employed, there was no overlap in FITC and DiI fluorescence. After formation of a tight seal (>1 G Ω) and compensation of pipette capacitance with amplifier circuitry, whole cell access (< 10 M Ω) was obtained by

rupturing the cell membrane for traditional whole cell patch recording or within 10–30 min for perforated patch recording. Cell capacitance was determined with five hyperpolarizing pulses (10 ms) from –60 to –80 mV. Whole cell capacitance and series resistance were compensated with the amplifier circuitry with series resistance compensation >80%. The average series resistance after compensation was $2.12 \pm 0.16 \text{ M}\Omega$. Neurons were held at –60 mV. The current-voltage (I–V) relationship for total K^+ current was determined with a series of 480 ms voltage steps to potentials between –90 and +60 mV in 10 mV increments that were initiated at 5 second intervals. Following establishment of stable I–V curves, one of three selective BK_{Ca} channel blockers IbTx, ChTx or paxilline was applied to each neuron for at least 1 min, while current stabilization in the presence of toxin was generally achieved in considerably less time; current was evoked with a 480 ms voltage step to +40 mV every 5 sec to monitor current stability. I–V protocols were evoked following current stabilization. The difference between current evoked before and after application of blocker was considered BK_{Ca} current. Of note, while we have previously described a Ca^{2+} -dependent K^+ current in a small subpopulation of DRG neurons (Gold *et al.*, 1996b), use of toxins to isolate BK_{Ca} current in the present study precluded cross contamination of BK_{Ca} currents described in the present study with other Ca^{2+} -dependent currents in DRG neurons. In order to determine reversal potential of evoked currents, a tail current protocol was employed before and after application of blockers. This protocol consisted of a 100 ms voltage step to +40 mV to activate currents, followed by a 50 ms step to voltages ranging between –100 and –30 mV. In a subset of neurons, a 40 ms pre-pulse to 0 mV was used to maximize Ca^{2+} influx, and therefore BK_{Ca} channel activation, prior to a test pulse. Leak subtraction (p/n) was not employed in any of the voltage-protocols. Capsaicin sensitivity was assessed after the completion of all other protocols. 500 nM capsaicin (Sigma, diluted in normal bath solution from a 1 mM stock solution made in 100% ethanol) was applied for ~1 sec with a glass pipette connected to a HSPC-1 (ALA Scientific Instruments, Inc. Westbury, NY) high speed pressure clamp pump system controlled by pClamp software. Neurons were considered capsaicin sensitive (CAP+) if capsaicin resulted in an inward current (>50 pA). This criterion was used based on control data in which the greatest spontaneous increase in holding current in response to the pressure application of vehicle bath (0.5% ethanol) was < 20 pA. Neurons were considered unresponsive (CAP–) if capsaicin failed to evoke the inward current.

Current clamp experiments were performed with standard whole cell patch configuration. To assess properties of the action potential waveform, a single action potential was evoked with depolarizing current injection (4-ms duration rectangular pulse at threshold intensity) and the following parameters were measured: action potential duration, overshoot, rates of rise and fall, AHP magnitude, and AHP decay rate. Overshoot was measured relative to 0 mV. Action potential duration (APD) was determined at 0 mV. The magnitude of the AHP was measured relative to the resting membrane potential. The decay of the AHP was fitted with a single exponential equation to determine the decay time constant. Maximal rates of action potential rise and fall were determined from the first derivative of the action potential waveform. Resting membrane potential (RMP) was determined immediately after establishing whole cell access. A ramp (250 ms) and hold (250 ms) depolarizing current injection protocol was used to assess action potential threshold (mV), current threshold (pA) and adaptation. The magnitude of the current injection during the ramp phase of this protocol was adjusted so that an action potential was evoked during the latter half of the ramp (i.e., > 125 ms) where the current amplitude obtained at the end of the ramp was maintained during the “hold” phase of the protocol. While this protocol does not facilitate comparisons between neurons with respect to the magnitude of each of these 3 parameters, it provides a sensitive way to assess the stability of these parameters over time within a cell and therefore the impact of BK_{Ca} channel blockers on these measures of excitability. Action potential (voltage) threshold was defined as the potential at which the action potential was initiated. This was determined by plotting the first derivative of voltage-trace versus time to determine the time at which dV/dt was significantly increased

above the mean of the dV/dt over the first 30 ms of the ramp. The Current threshold was defined as amount of current injected at the time of the time of action potential initiation. The total number of spikes evoked during the ramp and hold stimulus was used to measure of adaptation. Current clamp data were collected before and after application of IbTx to determine the contribution of BK_{Ca} currents to the action potential waveform and excitability.

Data analysis

Conductance-voltage (G - V) curves were constructed from I - V curves by dividing the evoked current by the driving force, such that $G = I/(V_m - V_{rev})$ where V_m is the potential at which current was evoked and V_{rev} is the reversal potential for the current determined from tail current protocols. Activation data were fitted with a Boltzmann equation of the form: $G = G_{max}/(1 + \exp[(V_{1/2} - V_m)/k])$, where G = observed conductance, G_{max} = the fitted maximal conductance, $V_{1/2}$ = the potential for half activation or availability, V_m = command potential and k = the slope factor. Because of the size of outward currents, I - V data were corrected for voltage-errors resulting from the uncompensated series resistance. Once G_{max} was determined, data were normalized with respect to G_{max} . G - V data from many neurons in which BK_{Ca} current appeared to activate in two distinct phases over different voltage ranges were poorly fitted with a single Boltzmann equation. These data were well fitted with a double Boltzmann equation of the form: $G = G1/(1 + \exp[(V1_{1/2} - V_m)/k1]) + G2/((1 + \exp[(V2_{1/2} - V_m)/k2]))$. In these neurons, total conductance was considered to be the sum of the maximal conductance attained during the first ($G1$) and second ($G2$) phase of current activation. BK_{Ca} currents (from I - V and tail current protocols) were fitted with a single exponential to obtain activation, inactivation and deactivation time constants. IbTx, ChTx or paxilline concentration-response data were fitted with a modified Hill equation of the form: Fractional inhibition = $[MAX_{inhib}/(drug\ concentration + IC_{50})]^n$ where MAX_{inhib} = maximal fractional inhibition; drug concentration = concentration of toxin; IC_{50} = half-maximal inhibitory concentration; n = Hill coefficient. A Students t -test or one-way ANOVA (with Holm-Sidak post-hoc test) was used to assess differences between subpopulations of cutaneous neurons with respect to properties of BK_{Ca} current. Mann-Whitney U and Kruskal-Wallis (with Dunn's post hoc test) were used when data sets were not normally distributed or of equal variance. Nevertheless, to facilitate comparisons between groups, data are presented as mean $\pm$ standard deviation unless otherwise noted in the text. Initial data analysis was performed with Clampfit (v 8.2, Molecular Devices), with subsequent analysis and curve fitting performed with SigmaPlot (v 10.0, Systat Software, Chicago, IL) and statistical analysis performed with SigmaStat (v 3.5, Systat Software).

Drugs

Paxilline was dissolved in 100% methanol, stored as a 1mM stock solution at $-20^{\circ}C$, and diluted in bath solution immediately prior to use. In control experiments on 5 IB4+ neurons, 1% methanol had no detectable influence on total outward current (data not shown). ChTx and IbTx were dissolved in bath solutions, stored as 1 μ M stock solution at $-20^{\circ}C$, and diluted in bath solution containing 0.05% BSA immediately prior to use. ChTx was obtained from Alomone Labs (Jerusalem, Isreal). IbTx used for the majority of experiments was also obtained from Alomone Labs. However, several milligrams were obtained from Sigma to determine whether toxin purity contributed to variability in BK_{Ca} current properties between DRG neurons. Similar results were obtained with the toxin obtained from either source. Paxilline was obtained from Sigma.

RT-PCR analysis

DRG from anesthetized male rats were harvested in a manner identical to that used for neuron isolation and plating. Trizol reagent (Invitrogen) was used to extract RNA which was done following manufacturer's protocol. First-strand cDNA synthesis was carried out using 1 μ g

total RNA with SuperScript II reverse transcriptase and an anchored oligo(dT)₁₂₋₁₈ primer from Invitrogen. All *rslo* primer sequences, with the exception of the “outer” primer set (see below), were based on Langer P. et al (2003) with some modification on primer length. *rslo* outer primers and β subunit primers were designed using PrimerQuest from IDT. PCR of β subunits were performed using 1 μ l of reverse transcription (RT) product in a total volume of 25 μ l with final concentration of 1X Titanium Taq PCR buffer (Clontech, Mountain View, CA), 0.2mM dNTPs, 0.4 μ M of each primer, 0.4 μ l of Titanium Taq polymerase. Amplification was carried out at 94°C, 1 minute, then, followed by 45 cycles at 94°C, 30 seconds and 68°C, 1 minute before a final elongation at 68°C for 4 minutes. A two step process was used for amplification of *rslo* splice variants. An “outer” primer set, spanning all 7 splice sites (Table 1), was used for a first round of amplification: 1 μ l of RT product in a total volume of 25 μ l with final concentration of 1X Takara LA buffer (Takara Bio Inc, Shiga, Japan), 2.5mM MgCl₂, 0.4mM dNTPs, 0.4 μ M of each outer primer, 1.25U of Takara LA Taq polymerase. The protocol for this first round of amplification consisted of 94°C for 1 minute, followed by 30 cycles of 94°C, 10 seconds, 68°C, 10 seconds, 72°C, 5 minutes, followed by a final elongation at 72°C for 10 minutes. The second round of amplification with splice variant specific primers was set up using the Titanium Taq polymerase as described in β subunits with 1 μ l of 1000 X dilution of the product from the first round of amplification. 5 μ l of PCR products were loaded onto 2% agarose/TAE gel.

Results

Concentration-response curves for three BK_{Ca} current blockers in cutaneous DRG neurons

The perforated patch configuration was used in initial experiments to minimize the impact of whole cell recording on resting levels of intracellular Ca²⁺ and endogenous Ca²⁺ regulatory mechanisms. In previous studies of BK_{Ca} currents in DRG neurons a single concentration of the specific BK_{Ca} channel blocker iberiotoxin (IbTx) was used to isolate BK_{Ca} current from the total outward current; results from these studies suggested that BK_{Ca} currents are present in a subpopulation of DRG neurons (Scholz *et al.*, 1998; Zhang *et al.*, 2003a). However, data from other cell types suggest that there is variability in IbTx sensitivity, with IC₅₀ values ranging from 250 pM in aortic smooth muscle cells (Galvez *et al.*, 1990) to 48 nM in nodose ganglion neurons (Christian *et al.*, 1994). Therefore, we assessed the concentration-dependence of IbTx-induced block of BK_{Ca} current in cutaneous DRG neurons. Since it was difficult to obtain stable recordings of K⁺ current in large diameter neurons for the amount of time required to complete a concentration response curve, concentration response data were only collected in small and medium diameter neurons. IbTx was applied over a 1000 fold concentration range (10nM, 30nM, 100nM, 1000nM and 10 μ M) to 16 neurons. Concentration-response data for each neuron was fitted with the modified Hill equation (see Methods) in order to obtain IC₅₀ and Hill coefficients (*n*). IbTx induced a concentration dependent reduction of total K⁺ current in 8 of 16 neurons with an IC₅₀ of 53.0 $\pm$ 32.0nM and a Hill coefficient of 1.31 $\pm$ 0.48. No concentration-dependent decrease in K⁺ current was observed in the other 8 neurons tested (i.e., see Fig 1A).

While the IbTx results suggested the presence of two populations of cutaneous DRG neurons (one with and one without BK_{Ca} current), there is evidence that the presence of the β 1-subunit results in a BK_{Ca} channel that is resistant to IbTx but still sensitive to ChTx (Dworetzky *et al.*, 1996; Zhou *et al.*, 1998). Therefore, the influence of ChTx was assessed. ChTx induced a concentration-dependent reduction in total K⁺ current in 5 of 12 neurons tested, with an IC₅₀ of 106 $\pm$ 70.4 nM and a Hill coefficient 1.45 $\pm$ 0.22 (Fig 1). However, in 7 of the 12 neurons studied, even the highest concentration of ChTx (10 μ M) had no detectable influence on total K⁺ current (i.e., see Fig. 1C).

Finally, there is evidence that the presence of the $\beta 4$ subunit results in a BK_{Ca} channel that is resistant to both IbTx and ChTx (Meera *et al.*, 2000). However, these channels are sensitive to paxilline (Hu *et al.*, 2001). Therefore, we assessed influence of paxilline in 7 neurons. Paxilline induced a concentration-dependent reduction in K^+ current in 3 of the 7 cutaneous DRG neurons tested with an IC_{50} of 106.7 ± 32.4 nM and a Hill coefficient of 1.01 ± 0.55 (Figure 1). In the other 4 neurons tested, paxilline had no detectable influence on total K^+ current, even at the concentration of $10 \mu M$ (i.e., see Fig 1E).

Variability in biophysical properties of BK_{Ca} current in cutaneous DRG neurons

In addition to the variability reported with respect to the pharmacological sensitivity of BK_{Ca} current (Reinhart *et al.*, 1989; Solaro *et al.*, 1995), there is evidence of variability in biophysical properties of BK_{Ca} currents both within cells in the same tissue (Lingle *et al.*, 1996; Dopico *et al.*, 1999) and between cells of different tissues (Hicks & Marrion, 1998). At least three major posttranscriptional mechanisms are involved in generating this diversity: alternative splicing of the α subunit, posttranslational modifications of the α -subunit and assembly of the α subunit with different β -subunits (Wallner *et al.*, 1998). While previous studies of DRG neurons suggest a single type of BK_{Ca} current is present, i.e. a rapidly activating non inactivating current (Scholz *et al.*, 1998), these studies were performed on unlabeled DRG neurons obtained from 3–9 day old rats. Therefore, we next assessed kinetics of BK_{Ca} current in cutaneous DRG neurons from adult rats.

BK_{Ca} current was isolated by digitally subtracting current obtained in the presence of blocker (Toxin Resistant) from that obtained in the absence of blocker (Total Current) (Figure 2A). IbTx (100 nM) was applied to 58 cutaneous neurons; and paxilline (1 μM) was applied to 62 cutaneous neurons. ChTx was not used in this and subsequent experiments because there are data indicating that ChTx is also able to block subtypes of voltage-gated K^+ currents (i.e., $K_{V1.3}$ (Kaczorowski & Garcia, 1999)). Neurons in which a toxin blocked 200 pA or more of the outward current evoked at +40 mV were considered to have BK_{Ca} current. This criterion was chosen based on the results of control experiments (5 small diameter IB4+ cutaneous neurons) in which current stability was monitored over time: 200 pA was > 2 times the largest decrease in current observed in the absence of toxin. There was considerable heterogeneity in the biophysical properties of BK_{Ca} current which was present in 71% (85/120) of cutaneous neurons studied (Figure 2B). Variability in activation rate, the presence of inactivation and the rate of deactivation was observed.

Analysis of the voltage dependence of activation revealed even more variability. Conductance-voltage ($G-V$) data for some cells were well fitted by a single Boltzmann equation, while data from others required a double Boltzmann equation. Typical $G-V$ curves of these two types of cutaneous DRG neurons are shown in (Figure 3).

Differential distribution of BK_{Ca} currents among subpopulations of cutaneous DRG neurons

1. Subpopulations of DRG neurons—Three criteria were used to define subpopulations of cutaneous DRG neurons: cell body diameter, IB4 binding (+ or –) and capsaicin (CAP) sensitivity (+ or –). Cell body size was used as a criterion based on data from analysis of cutaneous DRG neurons *in vivo* indicating that there is a rough correlation between cell body diameter and functional properties such that neurons with a small cell body diameter tend to have a high threshold for activation (i.e., are nociceptors) with slowly conducting axons while neurons with a large cell body diameter tend to have a low threshold for activation (i.e., are not nociceptors) with rapidly conducting axons (Fang *et al.*, 2002; Djouhri *et al.*, 2003; Fang *et al.*, 2005; Fang *et al.*, 2006). IB4 binding was used as a criterion based on anatomical and function evidence that IB4 labels a unique population of cutaneous nociceptive afferents, the vast majority of which have slowly conducting axons (Fang *et al.*, 2006), are devoid of the

neuropeptides calcitonin gene-related peptide and substance P (Zylka *et al.*, 2005), have unique termination patterns in the skin (Zylka *et al.*, 2005), and dorsal horn (Zylka *et al.*, 2005), appear to play an important role in the response to noxious thermal and mechanical stimuli (Rau *et al.*, 2009), and may play a particularly important role in signaling the affective, or emotional content of painful stimuli (Braz *et al.*, 2005). Finally, capsaicin sensitivity was used as a criterion because this compound is a selective agonist for the transient receptor potential vaneloid type 1 (TRPV1) receptor which is present in a unique subpopulation of nociceptive afferents (Caterina *et al.*, 1997) that may play a particularly important role on maintaining hypersensitivity associated with chronic pain (Karai *et al.*, 2004). Neurons were subdivided by cell body diameter into small (<30 μm), medium (30 to 40 μm) and large (>40 μm). All IB4+ cutaneous DRG neurons had a small cell body diameter. CAP sensitivity was assessed in a subset ($n = 107$) of the total number of cutaneous neurons studied, of which 38% (41 of 107) were CAP+. Of these, 33 had a small diameter and 8 had a medium diameter. No large diameter cutaneous DRG neurons were CAP+. Among 51 IB4+ neurons studied with CAP, 43% neurons were CAP+, on the other hand, among 41 CAP+ neurons, 54% neurons were IB4+. The distribution of these properties exemplifies the heterogeneity of DRG neurons.

2. BK_{Ca} current is differentially distributed in subpopulations of cutaneous DRG neurons—In the concentration-response studies described above, evidence of BK_{Ca} current was obtained in 16 of the 35 neurons studied with the 3 different channel blockers. Of these 16 neurons, 15 were IB4+ and 1 was IB4-. Similarly, 15 neurons had a small cell body diameter, and 1 neuron had medium cell body diameter compared to 3 and 16 small and medium diameter neurons, respectively, in which no BK_{Ca} current was detected. These differences in the distribution of BK_{Ca} current between subpopulations of cutaneous DRG neurons defined by IB4 binding (Fisher Exact test, $p < 0.01$) and cell body size (Fisher Exact test, $p < 0.01$) were statistically significant.

Consistent with the results obtained with the neurons used in the concentration-response analysis, use of 100nM IbTx, or 1 μM paxilline to assess the presence of BK_{Ca} current in a larger sample of cutaneous DRG neurons revealed a differential distribution among subpopulations of neurons. That is, there were statistically significant differences between subpopulations defined by IB4 binding and cell body diameter with respect to the distribution of BK_{Ca} current (Table 2). However, there was no statistically significant difference between subpopulations of cutaneous DRG neurons defined by CAP sensitivity with respect to the distribution of BK_{Ca} current (Table 2).

3. Biophysical properties of BK_{Ca} current among subpopulations of DRG neurons—Parameters describing the voltage-dependence of BK_{Ca} current activation and kinetics of activation, inactivation and deactivation were compared among subpopulations of cutaneous DRG neurons. Rates of activation, inactivation and deactivation were determined as described in Methods and illustrated in Figure 4. There were no significant differences between subpopulations of cutaneous DRG neurons with respect the kinetics of BK_{Ca} current activation or inactivation. However, the deactivation rate of BK_{Ca} current in both the total population of IB4- neurons as well as small diameter IB4- was slower than that of BK_{Ca} currents in IB4+ neurons. Results of this analysis are summarized in supplementary material Tables 1. Of note, while BK_{Ca} currents in a number of neurons were complex, and suggestive of the presence of several different channel types, tail currents were generally well fitted with a single exponential.

Analysis of the voltage-dependence of activation of BK_{Ca} current among subpopulations of cutaneous DRG neurons was somewhat complicated by the fact that it was necessary to use a double Boltzmann function to adequately fit G-V data from a subset of neurons. Nevertheless, two differences between subpopulations were observed. First, total BK_{Ca} conductance,

normalized to membrane capacitance, was larger in IB4+ than either the total population of IB4- neurons ($t_{(37)} = 3.61$, $p < 0.01$, unpaired t -test) or only those with a small cell body diameter ($t_{(28)} = 2.52$, $p = 0.018$, unpaired t test, supplementary table 2). These differences were not due to a differential distribution of low threshold BK_{Ca} current, as currents with a double-exponential G - V curve were equally distributed between subpopulations. And second, when only double Boltzmann data were compared, normalized BK_{Ca} conductance was again larger in IB4+ than in either the total population of IB4- neurons, or only IB4- neurons with a small cell body diameter (supplementary table 2). Interestingly, however, these differences were only detected with respect to the normalized conductance derived from the second component of the double Boltzmann (i.e., G_2). Because the concentration of IbTx (100 nM) used in the majority of experiments only blocked ~70% of total BK_{Ca} current and the concentration of paxilline used (1 μ M) should block > 90% of total BK_{Ca} current, only data from paxilline treated cells were included in this analysis. It should also be noted that there were no statistically significant differences between subpopulations with respect to the proportion of neurons with BK_{Ca} current fitted with a double Boltzmann function.

Low threshold, or T-type voltage-gated Ca^{2+} currents are present in a subpopulation of DRG neurons (Scroggs & Fox, 1992). This current is completely blocked by 0.5 mM amiloride. To begin to determine whether the low threshold component of the BK_{Ca} current observed in a subpopulation of DRG neurons is due to the presence of a T-type currents, BK_{Ca} currents were studied in a subpopulation of neurons before and after the application amiloride (0.5 mM). In 3 of 3 neurons studied with a low threshold BK_{Ca} current, amiloride had no detectable influence on the low threshold current (data not shown).

4. Density of K^+ current among subpopulations of cutaneous DRG neurons—

Current density was determined by normalizing peak current evoked at ~+20 mV (corrected for voltage-errors associated with uncompensated series resistance) with cell capacitance. There were statistically significant differences between subpopulations of neurons defined by IB4 binding and cell body diameter with respect to the density of total outward current, which was greater in IB4+ than in IB4- neurons, and in large-diameter neurons relative to small diameter neurons (supplementary table 3). Furthermore, consistent with the conductance data described above, BK_{Ca} current density was significantly larger in IB4+ than in IB4- cutaneous DRG neurons (Figure 5, supplementary table 3).

5. Source of Ca^{2+} underlying the activation of BK_{Ca} currents in DRG neurons—

Two additional experiments were performed in order to begin to identify the source of Ca^{2+} facilitating the activation of BK_{Ca} currents in DRG neurons. These experiments were performed with conventional whole-cell patch techniques to facilitate control over the intracellular milieu. Data was collected from 59 small diameter cutaneous neurons before and after the application of IbTx (100 nM). The distribution of BK_{Ca} currents among IB4+ and IB4- neurons was comparable to that obtained with perforated patch recording (e.g., toxin sensitive current was detected in 23 of 24 IB4+ neurons tested, but only 11 of 23 IB4- neurons ($p < 0.01$, Fisher Exact test)). To facilitate characterization of the relative contribution of Ca^{2+} influx via voltage gated Ca^{2+} channels in this series of experiments a two pulse protocol was used which consisted of a pre-pulse to 0 mV to facilitate Ca^{2+} influx, followed by a test pulse to +60 mV, a potential close to the reversal potential for Ca^{2+} (Fig 6 inset). Quantifying changes in total outward current evoked at +60 mV minimized the influence of changes in Ca^{2+} currents on total current. In the first series of experiments, Cd^{2+} (50 μ M) was added to the bath solution as a means of blocking Ca^{2+} influx via voltage-gated Ca^{2+} channels. In 5 of 5 IB4+ neurons tested, Cd^{2+} occluded the ability of IbTx to attenuate outward currents, a result significantly ($p < 0.01$ Fisher Exact test) different than that observed in the absence of Cd^{2+} where IbTx attenuated outward current in 23 of 24 IB4+ neurons tested (Fig 6). In the second series of experiments, EGTA in the electrode solution was replaced with the rapid Ca^{2+} chelator

BAPTA. IbTx had no influence on outward current evoked in 4 of the 7 IB4+ neurons studied with BAPTA ($p = 0.01$, Fisher Exact test, Fig 6). These last results suggest BK_{Ca} and voltage-gated Ca²⁺ channels are relatively tightly coupled in at least half the neurons studied.

Splice variants of the BK_{Ca} α subunit and accessory β subunits present DRG neurons

To begin to determine the extent to which heterogeneity in pharmacological and biophysical properties of BK_{Ca} currents in cutaneous DRG neurons may be due to a differential distribution of splice variants of the BK_{Ca} channel α subunit, *rslo*, and/or accessory β subunits, we analyzed BK_{Ca} channel mRNA obtained from L₄ and L₅ DRG harvested from 4 adult male rats. Following cDNA synthesis, PCR primers flanking 7 previously described splice sites in *rslo* (Brandle *et al.*, 2001; Langer *et al.*, 2003) were used to assess the presence of *rslo* splice variants in DRG (Fig 7). An example of the results from such an analysis is shown in Figure 7. Multiple splice variants were detected at 3 sites (X4, X6 and X7) with an alternative splice form the dominant band detected at X6. Comparable results were obtained with cDNA generated from all 4 rats studied. All 4 accessory β subunits were also detected in cDNA generated from whole ganglia (Fig 7).

Influence of BK_{Ca} currents on action potential waveform and excitability

To assess the functional consequences of BK_{Ca} current activation on the excitability of cutaneous DRG neurons, cutaneous DRG neurons were studied in current clamp mode before and after the application of IbTx. Preliminary data indicated that in the presence of 100 nM IbTx, the membrane potential would often fail to repolarize for hundreds of milliseconds following the upstroke of the action potential. This situation made it difficult to assess changes in excitability. Consequently, 50 nM IbTx was used in these experiments. Furthermore, because there were the most dramatic differences in BK_{Ca} current density in subpopulations of cutaneous DRG neurons defined by IB4 binding and between small and medium diameter DRG neurons, current clamp experiments were restricted to these populations of neurons.

The RMP in subpopulations of neurons defined by IB4 binding and cell body diameter were comparable (Table 3). APD in IB4+ ($n = 35$) and IB4- ($n = 15$) were also comparable (Table 3). APD was, however, significantly ($t_{(47)} = 2.99$, $p = 0.004$, unpaired t-test) longer in small ($n = 39$) than in medium ($n = 10$) diameter cutaneous neurons (Table 3).

IbTx-induced a significant increase in APD in small diameter and IB4+ cutaneous DRG neurons but not in medium diameter or IB4- cutaneous DRG neurons (group data shown in Table 3). Of note, IbTx prolonged the APD of all (35/35) IB4+ neurons tested but only 40% (6/15) of IB4- neurons tested ($p < 0.001$, Fisher Exact test). Typical recordings are shown in Fig. 8, before and after application of IbTx. IbTx had no significant influence on RMP (data not shown), overshoot and AHP magnitude in any of the subpopulations of cutaneous DRG neurons tested. However, IbTx significantly decreased AHP decay in IB4+ and small diameter neurons (Table 3). Application of IbTx also significantly decreased rate of action potential fall in IB4+ and small diameter neurons. Of note, even though there was no significant mean effect of IbTx on AHP amplitude, this parameter was reduced by 4.52 ± 1.12 mV in 16 of the 50 neurons tested.

A ramp and hold protocol (see methods) was used to assess IbTx mediated changes in excitability. IbTx (50 nM) was associated with a significant decrease in current threshold and an increase in the number of action potentials evoked in all groups of cutaneous neurons studied except those with medium diameter cell bodies (Table 4). Of note, IbTx-induced changes in excitability of IB4- cutaneous neurons appeared to reflect the influence of IbTx on a subpopulation, as no changes in excitability were observed in 66.7% (10/15) of IB4- neurons (typical recording shown in Fig. 8). In contrast, an IbTx-induced increase in excitability was

observed in all IB4+ neurons studied; this difference between IB4+ and IB4- neurons was statistically significant ($p < 0.01$, Fisher exact test). In 8 of 50 cutaneous DRG neurons recorded in current clamp, the relative impact of BK_{Ca} suppression on AHP and AP repolarization increased with prolonged depolarization, as APD became longer and AHP become smaller during a burst of action potentials in the presence of IbTx (Fig. 8).

Discussion

Perforated and conventional whole cell patch clamp techniques were used to characterize the pharmacological and biophysical properties of BK_{Ca} current among subpopulations of cutaneous DRG neurons from adult rats. Our primary findings were (1) BK_{Ca} currents are present in a significant fraction of cutaneous DRG neurons. (2) There is considerable heterogeneity in the biophysical properties of BK_{Ca} currents present in cutaneous DRG neurons which includes the presence of an inactivating BK_{Ca} current in a subpopulation of cutaneous DRG neurons, in addition to a persistent BK_{Ca} current previously described in DRG neurons (Scholz *et al.*, 1998). (3) There is a differential distribution of BK_{Ca} currents among subpopulations of cutaneous DRG neurons, most notably between IB4+ and IB4- DRG neurons. (4) Activation of BK_{Ca} currents in cutaneous DRG neurons is facilitated by Ca²⁺ influx via voltage-gated Ca²⁺ channels. (5) Multiple splice variants of the BK_{Ca} channel α subunit (*rslo*) and all 4 accessory β subunits were detected in mRNA extracted from whole DRG. (6) BK_{Ca} currents are involved in action potential repolarization and response adaptation to prolonged membrane depolarization in a subpopulation of putative cutaneous nociceptive afferents.

BK_{Ca} currents are present in a subset of cutaneous DRG neurons

BK_{Ca} currents were detected in ~ 71% of cutaneous DRG neurons studied. These results are consistent with previous studies in DRG neurons in which the distribution of BK_{Ca} current was determined with single channel recording and/or pharmacological techniques: DRG neurons lacking BK_{Ca} channels accounted for 33% of small and medium sized DRG neurons (Scholz *et al.*, 1998; Zhang *et al.*, 2003a), which was similar to 29% of all sized DRG neurons observed in the present study.

Additional experiments will be necessary to establish the basis for the variability between DRG neurons with respect to the pharmacological (i.e., toxin sensitivity) and biophysical properties of the BK_{Ca} currents. Nevertheless, there are two likely sources for this variability. One source arises from differences between subpopulations of DRG neurons with respect to the distribution of voltage-gated Ca²⁺ channels (Scroggs & Fox, 1992) and/or regulation of intracellular Ca²⁺ (Lu *et al.*, 2006). Although, our amiloride data argue against the presence of a low threshold, or T-type voltage-gated Ca²⁺ current as the cause of the low threshold BK_{Ca} currents detected in cutaneous DRG neurons. A more likely source, however, arises from variability in BK_{Ca} channel properties within and between neurons. BK_{Ca} channels are composed of the pore-forming α subunit (*rslo*) encoded by a single gene which may associate with at least one of four accessory β - ($\beta 1$ through $\beta 4$) subunits. Evidence that subunit composition influences pharmacological properties comes from heterologous expression studies suggesting that the co-expression of *rslo* with $\beta 1$ increases the IC₅₀ of IbTx from < 35 nM (α alone) to over 100 nM (Dworetzky *et al.*, 1996; Zhou *et al.*, 1998; Lippiat *et al.*, 2003). Co-expression of the *hslo* with $\beta 2$ has a smaller effect, increasing the IC₅₀ to >60 nM (Wallner *et al.*, 1999; Xia *et al.*, 1999). While results from these heterologous expression studies should be interpreted with caution because of the potential impact of overexpression on protein properties, the IC₅₀ of ChTx-induced inhibition of BK_{Ca} current in cutaneous DRG neurons is similar to that for inhibition of an inactivating BK_{Ca} channel in chromaffin cells in which $\beta 2$ subunits are thought to be present (Xia *et al.*, 2003). Evidence that subunit composition influences channel kinetics

also comes from heterologous expression studies where expression of the α -subunit alone, or the α -subunit in combination with the $\beta 4$ subunit results in a non-inactivating current, while the presence of $\beta 2$ or $\beta 3$ result in an inactivating current (Wallner *et al.*, 1999; Xia *et al.*, 1999; Brenner *et al.*, 2000). In addition, $\beta 1$ and $\beta 4$ have been reported to slow activation rate and deactivation rate of BK_{Ca} current (Lippiat *et al.*, 2003). Furthermore, there is evidence that the Ca^{2+} sensitivity and voltage dependence of different splice variants of the α -subunit (Lagrutta *et al.*, 1994; Tseng-Crank *et al.*, 1994), phosphorylation state of the α -subunit (Tian *et al.*, 2001; Widmer *et al.*, 2003) as well as the presence of different β -subunits (Wallner *et al.*, 1999; Lippiat *et al.*, 2003) may all influence Ca^{2+} sensitivity and therefore the voltage-dependence of channel activation. The presence of mRNA encoding multiple splice variants of *rslo* as well as all 4 β subunits is consistent with the suggestion that the variability in pharmacological and biophysical properties of BK_{Ca} current in cutaneous DRG neurons reflects, at least in part, the differential distribution of splice variants and/or β subunits among cutaneous DRG neurons.

Differential distribution of BK_{Ca} currents

BK_{Ca} currents appear to be preferentially expressed in IB4+ and small diameter cutaneous DRG neurons. Not only are BK_{Ca} currents more prevalent in these subpopulations of neurons but when comparing neurons in which BK_{Ca} current is present, it is present at higher density. The biophysical properties of the BK_{Ca} current in IB4+ neurons were different from those in IB4- neurons as well. Differences in channel density may account for the differences in current density. And differences in the regulation of intracellular Ca^{2+} (Lu *et al.*, 2006) may contribute to differences in both density and biophysical properties of BK_{Ca} currents among subpopulations of cutaneous DRG neurons. As discussed above, it is also likely that differential expression of splice variants, phosphorylation state and/or β -subunits contribute to these differences as well.

BK_{Ca} splice variants and β subunits

The presence of multiple splice variants of *rslo* have been well described (Brandle *et al.*, 2001; Langer *et al.*, 2003). And while the functional impact of differential splicing at the majority of these sites have yet to be described, the impact of differential splicing at the X4 site has been described in detail. This site, which corresponds to C2 described by others (Chen *et al.*, 2005), is the site of insertion of the 58/61 amino acid stress axis-regulated exon, or “STREX” insert. Previous evidence suggests the impact of STREX on the biophysical properties and second messenger regulation of BK_{Ca} currents is dramatic where the presence of STREX results in a ~25 mV hyperpolarizing shift in the activation of BK_{Ca} channels and more than a 2-fold decrease in the rate of channel deactivation (Xie & McCobb, 1998). Furthermore, in the absence of the STREX insert, stimulation of the cAMP second messenger cascade results in an increase in channel activity (Nara *et al.*, 1998), while activation of the same second messenger pathway in the presence of the STREX insert results in an inhibition of channel activity (Shipston *et al.*, 1999). The presence of channels with the STREX insert in nociceptive DRG neurons would enable this channel to contribute to the sensitization induced by inflammatory mediators such as prostaglandin E_2 , that utilize a cAMP second messenger cascade. Consistent with this suggestion, BK_{Ca} channels with a STREX insert could account of the fraction of total K^+ current inhibited by PGE_2 that has been described previously (Nicol *et al.*, 1997). An injury-induced increase in the proportion of channels with the STREX insert would facilitate this process.

The presence of all four β subunits in DRG was somewhat surprising given evidence that $\beta 1$ is not present in neural tissue (Jiang *et al.*, 1999). This raises the possibility that β subunits and splice variants detected in mRNA obtained from whole ganglia reflect the presence of BK_{Ca} channels in non-neural tissue such as vascular smooth muscle or satellite cells present in the

intact ganglia. That said, the presence of inactivating BK_{Ca} currents in cutaneous DRG neurons in the face of evidence that $\beta 2$ and $\beta 3$ subunits confer channel inactivation is consistent with the suggestion that the differential distribution of β subunits may contribute to the BK_{Ca} current heterogeneity observed. Furthermore, evidence for both inactivating and sustained BK_{Ca} currents in the same neuron suggests the presence of multiple channel configurations in the same neuron.

Physiological role of BK_{Ca} current in DRG neurons

Consistent with results from previous studies of BK_{Ca} currents in unlabeled sensory neurons (Scholz *et al.*, 1998; Zhang *et al.*, 2003b), our current clamp results indicate that BK_{Ca} currents serve two major functions in cutaneous neurons. One is the regulation of action potential duration and the second is the regulation of excitability. Thus, BK_{Ca} currents regulate the excitability of DRG neurons, and secondary to an influence on activity dependent changes in the concentration of intracellular Ca²⁺, would also influence Ca²⁺ dependent processes such as transmitter release, enzyme activity and transcription. The differential distribution and biophysical properties of these currents among subpopulations of cutaneous DRG neurons suggests that modulation of these channels would have a differential influence on afferent activity and therefore the impact of afferent activity on the central nervous system.

It has been suggested that the BK_{Ca} current mediated inhibition of action potential duration is a way of attenuating Ca²⁺ influx during an AP, thereby providing a mechanism for negative feedback on subsequent Ca²⁺ influx and hence transmitter release (Hu *et al.*, 2001). Given evidence suggesting that higher activity rates and consequently larger increases in intracellular Ca²⁺ appear to be necessary for the release of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) (Marvizon *et al.*, 1997), one might have predicted that a Ca²⁺ sensitive feedback mechanism such as that apparently mediated by BK_{Ca} currents, would be present in highest relative density in substance P and CGRP containing neurons. Yet the converse was observed in the present study as a distinguishing feature of IB4+ DRG neurons is that they do not, in general, express these peptides (Snider & McMahon, 1998). Thus, it is clear that an appreciation of the implications of the differential distribution of BK_{Ca} currents among cutaneous neurons will also require additional study. However, it is worth noting that recent evidence has highlighted a role for unique subsets of IB4+ neurons in nociceptive processing, particularly that associated with more troubling affective or emotional aspects of pain associated with tissue injury (Braz *et al.*, 2005). These data in the context of data from other lines of investigation indicating that there are both nerve injury (Baccei & Kocsis, 2000; Hogan *et al.*, 2000) and inflammatory mediator-induced decreases in voltage-gated Ca²⁺ current (Borgland *et al.*, 2002), raise the possibility that BK_{Ca} currents may play a significant role in persistent pain associated with tissue injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Concentration-dependent inhibition of K^+ current by BK_{Ca} channel blockers. K^+ current was evoked following a 500 ms voltage step to -100 mV with a 480 ms voltage step to $+40$ mV every 5 sec. Increasing concentrations of a single BK_{Ca} channel blocker was applied to each neuron for ~ 1 minute or until current stabilized. Examples of neurons in which BK_{Ca} channel blockers decreased (filled symbols) or had no concentration-dependent influence (open symbols) on K^+ current are shown in panels on the left. A, C and E are examples from neurons treated with iberitoxin (IbTx), charybdotoxin (ChTx) and paxilline, respectively. Peak outward current evoked at $+40$ mV was plotted as a percentage of current evoked over the 30 seconds prior to the application of a toxin. *Inset to A*: Total outward current evoked prior to

the application of IbTx (Trace 1 at time 1) and after the application of 10 μ M IbTx (Trace 2 at time 2). The voltage protocol used to evoke outward current is shown below the current traces. Concentration-response data from each neuron were normalized as a percentage of the maximal current inhibition obtained at the highest concentration of blocker applied. Pooled data from IbTx, ChTx and paxilline treated neurons are plotted as mean $\pm$ SEM in B, D and F panels, respectively. Data are fitted with a modified Hill equation as described in text in order to obtain values for IC₅₀ (concentration of blocker producing 50% inhibition of current). These values (as mean $\pm$ SD) were 53.0 $\pm$ 11.3 nM (n=7) for IbTx, 106 $\pm$ 31.5 nM (n=5) for ChTx, 106.7 $\pm$ 18.7 nM (n=3) for paxilline.

Figure 2.

Variability in kinetics of BK_{Ca} current activation, inactivation and deactivation in cutaneous DRG neurons. **A.** The same voltage-clamp protocol, consisting of 480 ms voltage steps ranging between -90 and $+60$ mV, following a 500 ms voltage-step to -100 mV from a holding potential of -60 mV, was used to evoke K⁺ current before (left traces, Total K Current) and after (middle traces, Toxin Resistant Current) application of paxilline ($1\mu\text{M}$) or IbTx (100 nM). IbTx was used in the example shown. BK_{Ca} current (right traces) was isolated by taking the difference between Total K⁺ Current and Toxin Resistant Current. Note the relatively slow rate of activation and absence of inactivation in the BK_{Ca} current present in this neuron. **B.** Examples of BK_{Ca} current isolated from 3 additional cutaneous DRG neurons as described in A. The currents on the left were isolated with paxilline, while the currents in the middle and right were isolated with IbTx. Note the relatively rapid rate of current activation in all three examples, as well as the presence of current inactivation in the middle and right traces and the relatively slow deactivation rate of the traces on the right. Time scale is the same in all.

Figure 3.

Variability in the voltage dependence of BK_{Ca} current activation. **A.** Examples of BK_{Ca} G–V data from two neurons. Data from one neuron was well fitted with a single Boltzmann function (open symbols), while data from the other could only be well fitted with a double Boltzmann function (closed symbols). G–V data were derived from I–V data as described in methods.

Data are normalized with respect to maximal conductance (G_{max}) or the total conductance (sum of fitted values obtained from the double Boltzmann function). **B.** Examples of G–V data from two neurons in which there were marked differences in the relative proportion of the first and second phase of BK_{Ca} current activation. Examples are typical of IB4⁺ neurons (closed symbols) and IB4[–] neurons (open symbols).

Figure 4.

Kinetics of BK_{Ca} current activation, inactivation and deactivation were estimated from the current evoked and isolated as described in Figure 2. **A.** The time constant for current activation was estimated with a single exponential fitted to the rising phase of the BK_{Ca} current. **B.** Current decay over the 480 ms voltage step was fitted with a single exponential equation in order to determine the time constant of current inactivation. **C.** Current decay following membrane repolarization to -90 mV from a voltage step to $+40$ mV was fitted with a single exponential equation in order to determine the time constant for deactivation.

Figure 5.

BK_{Ca} current density is larger in IB4+ than IB4- cutaneous DRG neurons. BK_{Ca} current evoked in cutaneous DRG neurons and isolated with paxilline (1 μ M) was normalized with respect to cell membrane capacitance. Data from IB4+ and IB4- neurons studied with conventional whole cell patch configuration in which paxilline blocked > 200 pA (at +40 mV) of current were pooled and plotted as mean $\pm$ SEM. I-V data were corrected for voltage errors associated with uncorrected series resistance. There was significantly more BK_{Ca} current in IB4+ than in IB4- cutaneous DRG neurons.

Figure 6.

BK_{Ca} current in cutaneous DRG neurons depends on Ca²⁺ influx via voltage-gated Ca²⁺ channels. BK_{Ca} I–V data evoked in IB4+ cutaneous DRG neurons with conventional whole cell patch configuration and isolated with iberitoxin (IbTx, 100 nM) was used to determine maximal conductance (G_{max}). G_{max} was normalized with respect to membrane capacitance. Normalized BK_{Ca} conductance, plotted as mean ± SEM, was significantly ($H_{(2)} = 18.18$, $p < 0.01$, Kruskal-Wallis One-Way ANOVA) attenuated when the Ca²⁺ chelator EGTA (11 mM) in the electrode solution was replaced by BAPTA (10 mM). IbTx induced inhibition of outward current was also completely occluded by the addition of Cd²⁺ (50 μM) to the bath solution as a means to block voltage-gated Ca²⁺ currents. *Inset*: Examples of outward current evoked in small diameter IB4+ cutaneous DRG neurons in which the additional of Cd²⁺ occluded the inhibitory actions of IbTx (top traces), which were readily apparent in the absence of Cd²⁺ (middle traces). The voltage protocol used to evoke currents is shown beneath the current traces.

Figure 7.

BK_{Ca} channel α subunit, *rslo* splice variants and β subunits in DRG: **A.** Schematic drawing *rslo* with possible splice sites in the cytoplasmic tail. Hydrophobic regions, 0–10, in the *rslo* sequence, are illustrated as cylinders. X2–X7 and SS5 indicate approximate location of potential splice sites. The numbers at each site indicate the expected size of PCR products in the absence of alternative splicing. **B.** RT-PCR was used to assess *rslo* and β subunits present in DRG. A nested PCR strategy described in Methods was employed in order to assess the presence of *rslo* splice variants. A single PCR product of the expected size for a “zero insert” was amplified at potential splice sites X2, X3, X5 and SS5. Multiple PCR products were amplified at splice sites X4, X6 and X7. mRNA encoding all four of the known BK_{Ca} β subunits were detected in whole DRG ganglion.

Figure 8.

Iberiotoxin (IbTx) caused a significantly larger increase in action potential duration and excitability in IB4+ than in IB4– cutaneous neurons. A single action potential was evoked with 4-ms depolarizing rectangular pulse (shown beneath voltage traces) at threshold intensity before (Baseline) and after (+IbTx 50 nM) the addition of IbTx. *Inset:* A ramp (250 ms) and hold (250 ms) depolarizing current injection protocol (shown beneath voltage traces) was used to assess action potential threshold (mV), current threshold (pA) and adaptation. A stable baseline (dashed trace) was obtained prior to the application of IbTx (50 nM). The results obtained with the small diameter IB4+ cutaneous DRG neuron shown in A, was typical of all IB4+ cutaneous neurons studied. Voltage traces shown for the small diameter IB4– cutaneous

DRG neuron shown in B, were typical of the 60% of IB4- cutaneous DRG neurons in which IbTx had no detectable influence on APD or excitability. Scale bars for the action potential in A is that same as that for B, while the scale bars in the inset in B is the same as that for the inset in A. The resting membrane potential for the neuron in A was -61 mV while that for the neuron in B was -58 mV.

Table 1
PCR Primers

Primer pairs are forward and reverse. A nested PCR strategy was used for amplification of *rslo*. First round amplification was performed with “Outer” primer pair. Second round amplification was performed with primer pair flanking putative splice sites, X2 though X7. *rslo* splice sites, X2 through X7 are based on the nomenclature used by Langer et al (2003).

Target (accession #)	Primer sequence	Product: Expected Observed
Rslo Outer (U40603)	CAATGTCTACAGTGGGTACGGG	
	GTGGAGGAGGCCTACCATGAGAAC	
Rslo X2	GGCCATGTTTGCCAGCTACGTC	115, 208
	TGTGCCCACAGACTACAATGTGC	115
Rslo X3	TAAGCTCCTGATGATAGCCATTGAG	138
	CACCTGCGATGAAAAATCCTAAAGTA	138
Rslo X4	GTCATGATGACGTCACAGATCCC	115, 298
	CCTCATGCCCCATTACGTTGTT	115, 225, 275, 390
Rslo X5	TGAAGATGAGCAACCGCCAACAC	138
	CATGTTGTCAATCTGATCGTTGCC	138
Rslo X6	CCTCCTGGAATGGACAGATCATC	161, 242
	TGTGTCAGGGTCATCATCATCATC	161, 242, 400
Rslo SS5	AGTTTGAACCTGTACCGACAGACC	165
	CGGTTTGGCCGATTGTGCTGTGG	165
Rslo X7	AGTCCTCTGTCCACTCCATCC	168
	GGCTTGGAATTTGAATGTTTCTGGG	168, 210
Beta1 U54498	TGCCGCCATCACTTACTACATCC	445
	TGATAAGAAGGCCACCAGTCAGC	445
Beta2 NM_176861.1	ACGTGAACCTGACATCTTCTGGG	219
	GCCAGAAGAGAGAATGGAACAGC	219
Beta3 AB297662	AGGGATCATCTGCTCAACAGTGC	190
	AAACCAACAATCAGAGCGCCTCC	190
Beta4 AB050637	ACGTGAACAACCTCCGAGTCCAAC	359
	TGCGTTTCTTCATGGCTTCTGCC	359

Table 2
Distribution of BK_{Ca} currents among subpopulations of Cutaneous DRG neurons

A neuron was considered to have BK_{Ca} current if iberiotoxin (IbTx) or paxilline inhibited >200 pA of outward current evoked at +40 mV. Small, medium and large refer to cell body diameter where small is < 30 μ m, medium is between 30 and 40 μ m, and large is >40 μ m. CAP is capsaicin, were neurons were considered CAP+ if 500 nM capsaicin evoked an inward current > 50 pA. N (total) refers to the total number of neurons studied in each subpopulation. % with BK_{Ca} current refers to the percentage of neurons in each subpopulation in which BK_{Ca} current was detected. There were significant differences between groups defined by IB4 binding ($\chi^2 = 24.46$ with 1 degree of freedom, $p < 0.001$) and cell body size ($\chi^2 = 19.68$ with 2 degrees of freedom, $p < 0.001$) with respect to the percentage of neurons in each subpopulation with BK_{Ca} current. Of note, because multiple criteria were used to define subpopulations, data from the same neuron may be included in multiple rows.

Subpopulation	N (total)	% with BK _{Ca} current
IB4+	54	94
IB4−	66	52
Small	76	83
Medium	30	40
Large	17	76
CAP+	32	68
CAP−	75	70

Table 3
Influence of BK_{Ca} current inhibition on action potential waveform

RMP: resting membrane potential prior to the application of IbTx; ADP: action potential duration; AHP amplitude: magnitude of the afterhyperpolarization, relative to resting membrane potential; AHP decay: time constant of the decay of the afterhyperpolarization; Maximum rate of rise: determine from the first derivative of the action potential waveform; Maximum rate of fall: determined with the maximum rate of rise; Overshoot: magnitude of the action potential above zero mV. Data are presented as mean ± standard deviation.

Active Property	IbTx	Subpopulation			
		IB4+ (n=35)	IB4- (n=15)	Small (n=39)	Medium(n=10)
RMP(mV)		57.4 ± 8.1	58.1 ± 8.4	57.4 ± 7.9	62.4 ± 4.2
APD(ms)	Before	5.7 ± 3.0	4.1 ± 4.6	5.8 ± 3.7	2.2 ± 1.6 [#] t = 2.99
	After	13.2 ± 13.6 [*] t = -3.50	4.2 ± 4.6	11.9 ± 12.5 [*] t = -3.35	2.7 ± 2.5
AHP Amplitude (mV)	Before	12.9 ± 5.9	17.8 ± 8.1 [#] t = -2.40	13.9 ± 6.2	17.0 ± 10.8
	After	15.2 ± 7.1	25.0 ± 6.6	16.7 ± 6.9	26.3 ± 25.9
AHP decay (τ ms)	Before	60.4 ± 59.2	83.8 ± 126	91.9 ± 182	63.1 ± 77.4
	After	25.7 ± 17.2 [*] t = 3.66	69.7 ± 95.6	46.8 ± 72.4 [*] t = 2.31	53.6 ± 77.8
Maximum rate of rise	Before	131.4 ± 56.2	172.1 ± 128	147.7 ± 94.9	199.5 ± 113
	After	111.9 ± 62.1	185.0 ± 207	132.6 ± 127	139.3 ± 108
Maximum rate of fall	Before	98.4 ± 91.1	103.0 ± 41	104.1 ± 83.5	113.7 ± 43.5
	After	66.6 ± 91.1 [*] t = 2.06	90.7 ± 62	64.8 ± 7.1 [*] t = 4.82	88.1 ± 27.5
Overshoot (mV)	Before	39.6 ± 8.3	42.7 ± 5.4	40.3 ± 8.1	42.4 ± 2.8
	After	40.1 ± 8.3	40.2 ± 8.1	40.3 ± 7.5	39.3 ± 9.2

* indicates significant difference (paired t-test p < 0.05) between before and after 50nM IbTx application.

[#] indicates significant difference (unpaired t-test, p < 0.05) between IB4+ and IB4- or small and medium diameter cutaneous DRG neurons; t values are shown below statistically significant differences.

Table 4
Influence of BK_{Ca} current inhibition on the excitability of cutaneous DRG neurons

Current and voltage threshold were determined, as described in Methods, from the response to the current injected during a ramp depolarization that was associated with action potential generation. Number of spikes was the total number of spikes evoked during a ramp (250 ms) and hold (250) stimulation protocol. Data are presented as mean ± standard deviation.

Subpopulation	Current Threshold (pA)		Voltage Threshold (mV)		Number of Spikes	
	Before IbTx	After IbTx	Before IbTx	After IbTx	Before IbTx	After IbTx
IB4+ (n=35)	316 ± 238	211 ± 144* t = 5.25	-17.0 ± 7.6	-18.4 ± 5.9	1.6 ± 1.1	4.4 ± 2.5* t = -5.45
IB4- (n=15)	224 ± 151	131 ± 87* t = 2.63	-22.3 ± 11.6	-19.4 ± 8.9	1.5 ± 1.2	5.3 ± 6.6* t = -2.27
Small (n=39)	338 ± 227	202 ± 139* t = 5.0	-17.3 ± 5.4	-17.6 ± 5.5	1.5 ± 1.2	4.6 ± 5.6* t = -5.18
Medium (n=10)	235 ± 212	113 ± 69	-27.0 ± 15.3# t = 3.31	-23.8 ± 11.6	2.3 ± 1.9	4.5 ± 10.1

* indicates significant difference between before and after 50nM IbTx application (paired t-test, p < 0.05).

indicates significant difference between IB4+ and IB4- or small and medium diameter cutaneous DRG neurons (unpaired t-test, p < 0.05). t values are shown below statistically significant differences.