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SODIUM CURRENTS OF LARGE (A β -TYPE) ADULT CUTANEOUS AFFERENT DORSAL ROOT GANGLION NEURONS DISPLAY RAPID RECOVERY FROM INACTIVATION BEFORE AND AFTER AXOTOMY

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Abstract

Voltage-dependent Na-currents were studied, using whole cell voltage clamp, in acutely dissociated, large (mostly A β -fiber type) cutaneous afferent dorsal root ganglia neurons (L₄ and L₅) from the adult rat. Cells were dissociated 14–17 days after axotomy. Control and axotomized neurons were identified via the retrograde marker hydroxy-stilbamide (fluorogold) which was injected into the lateral and plantar region of the skin of the foot and were studied using whole cell patch clamp techniques within 12–20 h of dissociation and plating. Cells were dissociated 14–17 days after injury. Both control and axotomized neurons displayed complex Na-currents composed of components with distinct kinetic and pharmacological properties. The large (48–50 μ m diameter) control cutaneous afferent neurons, many of which likely give rise to myelinated A β -fibers, exhibited Na-currents with both slow and fast inactivating kinetics. The fast inactivating current in large cutaneous afferent dorsal root ganglion neurons was tetrodotoxin-sensitive and recovered from inactivation ~four-fold faster at –60 mV ($P < 0.001$) and ~six-fold faster at –70 mV ($P < 0.001$) than the tetrodotoxin-sensitive current in small (< 30 μ m diameter) neurons. Further, while the tetrodotoxin-sensitive currents in smaller dorsal root ganglion neurons (mainly C-fiber type) reprime approximately four-fold faster following peripheral axotomy, repriming of the fast inactivating current in larger cutaneous afferent neurons was not significantly altered following axotomy. However, while 77% of control large neurons were observed to express the slower inactivating, tetrodotoxin-resistant current, only 45% of these large neurons did after axotomy.

These results indicate that large adult cutaneous afferent dorsal root ganglion neurons (A β -type) express tetrodotoxin-sensitive Na-currents, which have much faster repriming than Na-currents in small (C-type) neurons, both before, and after axotomy. Like small neurons, the majority of large neurons downregulate the tetrodotoxin-resistant current following sciatic nerve section.

Keywords

axotomy; nerve crush; sodium channels; cutaneous afferents; fast repriming current; tail currents

Excitability in dorsal root ganglion (DRG) neurons is substantially altered following axotomy, and it has been suggested that this may contribute to the molecular pathogenesis of many

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chronic pain syndromes associated with injury of spinal sensory neurons (Cummins and Waxman, 1997; Devor, 1994; Rizzo et al., 1995). A number of workers have demonstrated Na^+ (Cummins and Waxman, 1997; Dib-Hajj et al., 1996; Rizzo, 1997; Rizzo et al., 1995) and K^+ (Everill et al., 1999; Everill and Kocsis, 2000) channel and GABA_A (Oyelese et al., 1995; Oyelese and Kocsis, 1996) receptor reorganization in cutaneous (DRG) neurons after axotomy, which may contribute to alterations in excitability induced by nerve injury.

DRG neurons express both fast activating, fast inactivating tetrodotoxin-sensitive (TTX-S) Na-currents, and slow inactivating tetrodotoxin-resistant (TTX-R) Na-currents (Caffrey et al., 1992; Elliott and Elliott, 1993; Kostyuk et al., 1981; Roy and Narahashi, 1992; Scholz et al., 1998). Recently, Cummins and Waxman (1997) demonstrated that in small (C-type) spinal sensory neurons of 18–25 μm diameter, TTX-R Na^+ currents were downregulated and a rapidly repriming TTX-S Na-current was upregulated after axotomy within the sciatic nerve. The effects of axotomy on larger DRG neurons of 40–50 μm have initially been examined by Rizzo et al. (1995). They demonstrated that control, i.e. non-axotomized, neurons tended to have both a fast and slow Na-current which suggested that the large neurons have at least two kinetic varieties of channel. In these larger cells axotomy induced a number of changes. Kinetically slow TTX-R Na-current was significantly attenuated, or removed; and a singular, TTX-S kinetically fast form of Na-current was seen to predominate. However, Rizzo et al. (1995) did not provide quantitative data on the changes in Na-current amplitudes following peripheral axotomy. Furthermore, it is not known if the TTX-S current in larger neurons exhibits slow recovery from inactivation, as has been observed in control C-type neurons, and if axotomy alters the repriming kinetics of the TTX-S currents in large neurons, as it does in the mainly C-fiber type neurons. Since not *all* small type neurons have C-fibers, throughout this paper, the term 'C-type' neurons has been used to mean 'mainly C-fiber type' neurons; and since not *all* large neurons have A β -fibers the term 'A β -type' has been used to mean 'mostly A β -fiber type'.

Repriming kinetics may be an important determinant of repetitive firing properties and neuronal excitability (Cummins and Waxman, 1997). Therefore, we examined the differences in properties of Na-currents in large cutaneous A β -type neurons, a large proportion of which give rise to myelinated axons involved in tactile sensation of the skin, before and after sciatic nerve ligation. We observed significant differences compared to Na-currents in small C-type neurons, many of which are involved in nociception and temperature sensation. Differences between the kinetic properties of the Na-currents in small and large neurons before and after nerve injury may provide a clue as to what part these currents may play in the modulation of hyperexcitability, which contributes to pain. Part of this work showing that recovery from inactivation is faster following axotomy in large DRG neurons has been presented in abstract form (Everill et al., 1999).

EXPERIMENTAL PROCEDURES

Cell identification and culture techniques

Cutaneous afferent cells were identified using retrograde fluorogold labelling in adult female Wistar rats (180–240 g; Harlan, Chicago, IL, USA). Fluorogold solution (2–4%; Molecular Probes, Eugene, OR, USA) in distilled water was injected into the skin of the lateral foot. Every effort was made to limit labelling to cutaneous afferents; injections into the lateral plantar region have been shown to expose cutaneous afferents; cells with distinctly different kinetics to those of muscle afferents (Honmou et al., 1994; Oyelese et al., 1995; Oyelese and Kocsis, 1996). One week later the sciatic nerve was exposed and ligated (4–0 silk suture; AR-Med Ltd, Bracknell, UK) near the sciatic notch bilaterally (Kocsis et al., 1984). To prevent regeneration the nerve was sectioned immediately distal to the ligature site; an approximate 10 mm section of the distal nerve was removed and the distal stump was retracted. A silicone cap was sutured

to the end of the proximal stump. The efficacy of ligation of the sciatic nerve and its relationship to the numbers of axotomized neurons has some variability, up to 30% of the DRG neurons at L4 and L5 may remain unaffected by this procedure (Himes and Tessler, 1989). Three weeks post-injection, and 2 weeks post-ligation, the rats were exanguinated under pentobarbital anesthesia (60 mg/kg; i.p.) and lumbar ganglia (L₄, L₅) excised and prepared for dissociation and culture (Honmou et al., 1994). Standard cell culture procedures were followed (Oyelese et al., 1995). Briefly, the L4 and L5 DRG were harvested from adult female Wistar rats. The DRG were treated with collagenase A (1 mg/ml) for 25 min, and collagenase D (1 mg/ml) with papain (30 U/ml) for 25 min, dissociated in Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 10% fetal bovine serum and plated on glass coverslips. Recordings were made within 24 h of dissociation. Results are reported from 57 identified adult cutaneous afferent DRG neurons taken from 29 rats. The cutaneous afferent neurons, large, 48–50 μ m in diameter, were identified *in vitro* by fluorescence on brief exposure of the culture dishes to ultraviolet light. This technique has been shown to reveal cutaneous afferent neurons with distinctly different kinetic and pharmacological properties to those of muscle afferents (Honmou et al., 1994; Oyelese et al., 1995; Oyelese and Kocsis, 1996). The majority of DRG cells were studied after short-term culture (12–24 h). The short-term culture provided cells with truncated axonal processes that can be readily and reliably voltage clamped allowing the cells sufficient time to adhere to the glass coverslip. The spontaneous electrical activity characteristic of DRG neurons following nerve injury can be observed in isolated injured neurons but not in isolated control neurons (Study and Kral, 1996), indicating that dissociation does not drastically alter the electrophysiological properties of the DRG neurons. Furthermore, adult rat DRG neurons maintained *in vitro* for 24 h display a profile of sodium channel mRNA expression similar to that for DRG neurons *in situ*, indicating that short-term culture does not substantially alter the expression of sodium channel mRNAs in these cells (Black et al., 1996). These results suggest that at less than 24 h in culture changes in Na-current properties are minimal. By contrast, changes in both Na-currents and sodium channel mRNA expression can be seen after 7 days *in situ* (Fjell et al., 1999), clearly demonstrating that long-term culture can significantly alter the electrical properties of DRG neurons. It should be noted, however, that Liu et al. (2000a) have reported that spontaneous firing in myelinated DRG neurons *in vivo* sharply increases 16–24 h following spinal nerve injury, suggesting that some changes in electrical properties could have taken place following dissociation. To address this possibility, we examined some cells 2–5 h after dissociation.

All experimental procedures conformed to the NIH Guide for Care and Use of Laboratory Animals and the Yale University Animal Care and Use Committee. Every effort was made to minimize animal suffering and to reduce the number of animals used.

Electrophysiological techniques and analysis

The whole cell patch clamp method (Hamill et al., 1981) was used to record voltage-dependent Na-currents from the cultured DRG neurons. Patch electrodes were of 1.0 to 1.8 M Ω . Cells chosen for recording were between 20 and 30 μ m for the small neurons and between 48 and 50 μ m for the large neurons. In order to minimize changes caused by altered transcription of ion channel genes that could be triggered by dissociation electrophysiological measurements were obtained 12–20 h after dissociation and plating. The neurons were held at –80 mV and various stimulation protocols were applied. Coverslips plated with the DRG neurons were rinsed with normal bath solution (see: Table 1, E2) and placed in a recording chamber on the stage of an inverted phase-contrast microscope (Nikon, Diaphot, Melville, NY, USA), and perfused with solution. Recording electrodes were fabricated from thin-walled, single filamented, borosilicate glass tubing (Warner Instrument Corp., Hamden, CT, USA), with a micropipette puller (Model P-97, Sutter Instruments, CA, USA), and fire-polished with the use

of a Narashige MI 83 microforge, to a resistance of 1–1.8 M Ω seal resistances were ≥ 1 G Ω . All recordings were made at room temperature ($21 \pm 1^\circ\text{C}$).

Voltage clamp recordings were made in the whole cell patch clamp configuration (Hamill et al., 1981) using an EPC9 amplifier driven by the 'Pulse' program (Heka-Elektronik, Germany). The neurons were voltage clamped at -80 mV for all experimental manipulations. Capacity and leakage subtraction were performed using a $p/ \pm 6$ subtraction protocol. Series resistance compensation of 50–75% was routinely employed to reduce any voltage error. Cells in which series resistance compensation was possible but less than 50% were discounted from the study. Large cell capacitance was standardized at 90 ± 10 pF. The current was sampled at 50 kHz, low-pass filtered (four-pole Bessel) at 10 kHz, unless otherwise stated, and initially digitized and stored on computers (Macintosh, G3 Blue and White, and PowerBook 1400c). Where necessary, currents were zero-subtracted (DC leak current based on the data points corresponding to the first stored segment are subtracted to zero) (Everill et al., 1998; Everill and Kocsis, 1999, 2000). Exponential fits via the Heka program for Macintosh that uses the root mean square (RMS) deviation between fit and data were applied to estimate inactivation time constants. The RMS value is used as residual for the simplex fit algorithm (see Heka Patch Clamp EPC9, Pulse Fit Manual 8.0, 1997). Statistical analysis was by way of a paired two sample Student's *t*-test for mean. A probability level of < 0.05 was set for statistical significance.

Separation of fast and slowly inactivating Na⁺ currents in large cutaneous afferents

Previous studies have shown that in both small DRG (Cummins and Waxman, 1997) and large cutaneous afferents (Renganathan et al., 2000) the fast inactivating Na-currents are TTX-S and the slowly inactivating Na-currents are TTX-R. The slowly inactivating Na-current in both small and large cutaneous afferent neurons has been shown to be produced by the sensory neuron-specific (SNS) TTX-R sodium channel (Cummins et al., 1999; Renganathan et al., 2000). We have never observed TTX-R fast inactivating sodium channels in DRG neurons. Thus fast inactivating and slowly inactivating Na-currents in DRG neurons can be separated using 300 nM TTX. However, prepulse inactivation and digital subtraction could also be used to separate fast inactivating and slowly inactivating Na-currents in DRG neurons (Roy and Narahashi, 1992; Cummins and Waxman, 1997; Renganathan et al., 2000). We used this later approach because it is quicker than TTX subtraction. Prepulse inactivation takes advantage of the differences in the inactivation properties of fast and slowly inactivating Na-currents (Roy and Narahashi, 1992). At hyperpolarized holding potentials (from -130 to -100 mV) fast inactivating and slow inactivating Na-currents are fully available for activation, however at depolarized potentials (-50 to -40 mV) fast inactivating channels are largely inactivated and, therefore, only slowly inactivating Na-currents are available for activation. In order to measure the amplitude of the fast inactivating and slowly inactivating Na-currents, neurons were held at -80 mV, prepulsed to holding potentials ranging from -130 to -10 mV in 5 mV steps for 500 ms, followed by a 20 ms test pulse to -10 mV to elicit Na-current. Subtraction of the slowly inactivating Na-currents, obtained following the prepulse to ~ -50 mV, from Na-currents elicited following more hyper-polarized prepulse potentials yields fast inactivating Na-currents.

Solutions

To reduce current amplitude and in an attempt to reduce errors caused by series resistance artifacts, tetramethylammonium chloride (TMA) was used as the primary non-permeant monovalent cation and only 20 mM Na⁺ was used in the extra-cellular solution. Cs⁺ salts were used internally in order to minimize current through voltage-dependent K⁺ channels. The standard bath solution contained in (mM) NaCl 20, TMAcI 115, KCl 3, CaCl₂ 1, MgCl₂ 1, CdCl₂ 100 μM , HEPES 10. Extracellularly osmolarity was adjusted to 305–310 mosmol using

glucose, and pH was adjusted to 7.4. Electrodes were filled with (in mM): NaCl 10, CsCl₂ 125, EGTA 1, MgCl₂ 2; osmolality was adjusted to 300–305 mosmol with glucose, and the pH was adjusted to 7.2.

RESULTS

Na-currents were recorded from relatively large A β -type (48–50 μ m diameter) cutaneous afferent neurons and compared to Na-currents from small C-type (< 25 μ m diameter) neurons. Large neurons of this type give rise to myelinated axons relating to skin. However, we did not distinguish between high and low threshold mechanoreceptors. These large cutaneous afferent DRG neurons express both slowly inactivating, TTX-R (SNS-like) current, and fast inactivating TTX-S current.

Representative waveforms, typical of the control neurons, can be seen in Fig. 1A. Both fast inactivating and slowly inactivating Na-currents can be observed in the majority of these neurons (77%). The neurons were initially held at –80 mV and then stepped down to –130 mV for 500 ms (conditioning prepulse potential), then stepped up to 0 mV for 20 ms (stimulation pulse potential) to initiate the spike, then returned to the holding potential (Fig. 1D). In the sequential pulses the prepulse was increased in 5 mV steps up to –5 mV (Fig. 1D). This protocol can be used to estimate the amplitudes of the fast and slowly inactivating Na-currents in DRG neurons (Cummins and Waxman, 1997). Figure 1C shows the normalized curves of the steady-state voltage inactivation for the traces in A and B.

Fourteen days following axotomy the amplitude of slowly inactivating, TTX-R current was greatly decreased, or extinguished, in large cutaneous afferent neurons (Fig. 1B). Fifty-five percent ($n = 15$) of the large cutaneous afferent neurons ($n = 29$) expressed only fast inactivating Na-currents following axotomy; the average current in these cells also being largely reduced compared to controls ($n = 28$) in those cells where it was still detectable. In controls 29% ($n = 8$) of cells expressed only fast inactivating current. A similar decrease in the proportion of cells expressing slowly inactivating Na-currents was reported by Rizzo et al. (1995). As can be seen in Table 2, the amplitude of the slowly inactivating current decreased by more than 60% following axotomy. However, while Rizzo et al. (1995) suggested that the amplitude of the fast inactivating currents might increase following axotomy, we observe a slight decrease in the amplitude of fast inactivating TTX-S current following axotomy (Table 2).

As can be seen in both Fig. 1A and B, inward currents are also generated when the large cutaneous afferent neurons are repolarized to –80 mV. These large tail currents were recorded in 88% of cells examined. Because these currents were observed in axotomized neurons that expressed only fast inactivating current as well as in control neurons, it is likely that this current is not dependent on the SNS sodium channel which underlies the TTX-R slowly inactivating Na-current. Therefore, we hypothesized that these tail currents might be generated by the channels that underlie the fast inactivating Na-currents. This hypothesis was tested by adding 250 nM TTX to the bath, which blocks the fast Na-currents in these cells. Figure 2A_i (control) shows a family of currents from an axotomized large cutaneous afferent DRG neuron before presentation of 250 nM TTX. Typically, as with a large proportion of ligated cells, no slow current is visible; only fast activating/inactivating current. The peak tail currents in this cell are approximately 5 nA. In Fig. 2B_i, after 3 min of 250 nM TTX, all of the fast current was blocked. The tail current, however, is left untouched, indicating that it is not generated by the same channels that underlie the fast inactivating Na-current ($n = 7$). This lack of effect on the tail current is validated by a further protocol, examining just the tail currents in a cell lacking expression of slow current, as seen in the two lower panels, A_{ii} (control) and B_{ii} (250 nM TTX), of Fig. 2. In these recordings the depolarizing pulse was kept constant and the repolarizing

pulse ranged from -10 mV to -90 mV (see pulse protocol bottom right). No difference was observed in the tail currents before (A_{ij}) and after (B_{ij}) TTX application.

We also examined the kinetics of recovery from inactivation of the TTX-S Na-currents in large (Fig. 3A) and small (Fig. 3B) neurons. The currents in Fig. 3A and B were elicited using a prepulse to -20 mV for 20 ms to inactivate the current, then brought back to -80 mV for increasing recovery durations prior to a test pulse of 0 mV (Fig. 3D). The ms values (arrows) indicate the recovery duration for a large A β -type (Fig. 3A) and a small C-type (Fig. 3B) neuron. The TTX-S current had a time constant for recovery from inactivation of approximately 25 ms at -80 mV for the A β -type neuron, and approximately 150 ms for the C-type neuron. Figure 3C is a graphical representation comparing the difference in the repriming time course of fast inactivating Na-current at -80 mV for the data shown in Fig. 3A and B. The duration of recovery is plotted against fraction of current recovered and shows a clear difference in recovery from inactivation kinetics between large and small neurons.

Axotomy induced changes in the Na-currents expressed in large cutaneous afferent DRG neurons. Axotomy by peripheral nerve section and ligation in large neurons results in faster recovery from inactivation, as can be seen in Fig. 4A, where time constants of recovery are plotted at different recovery voltages for control and axotomized large neurons. Time constants were obtained using the pulse protocol as seen in Fig. 1. At -80 mV the fast inactivating current in large axotomized neurons recovered from inactivation $\sim 20\%$ slower than the fast inactivating current in large control neurons. This difference, although relatively small, was significant ($P < 0.05$). However, there was not a significant difference at -70 or -60 mV, potentials that are probably more physiologically relevant to an A β -type neuron *in vivo*. We were not able to reliably measure recovery at -50 mV, because of the small size of the TTX-S currents at this potential. This may be caused in part by the 5–10 mV negative shift in voltage dependence that can occur in the whole cell recording configuration.

Previously, we reported that the fast inactivating current in control small neurons recovers slowly from inactivation ($\tau \sim 150$ ms at -80 mV), but that following axotomy the recovery is more rapid ($\tau \sim 40$ ms at -80 mV) (Cummins and Waxman, 1997; Black et al., 1999). A comparison of time constants of recovery in large A β -type cutaneous neurons with that of small neurons (Black et al., 1999) is shown in Fig. 4B. The TTX-S current in control large A β -type cutaneous afferent DRG neurons ($n = 21$) recovered from inactivation $\sim$ four-fold faster at -60 mV ($P < 0.001$) and $\sim$ six-fold faster at -70 mV ($P < 0.001$) than the TTX-S current in control small (< 30 μ m diameter) DRG neurons ($n = 12$) (Fig. 4B). Following axotomy, the Na-currents in large cutaneous afferents recover from inactivation $\sim$ three to four-fold faster at -70 and -60 mV than Na-currents in small DRG neurons.

This suggests that the fast inactivating current in large cutaneous afferent neurons reprimers much faster ($\tau \sim 20$ ms at -80 mV) than the fast inactivating current in small DRG neurons. However, the Na-currents in small, control, DRG neurons were recorded with 140 mM extracellular sodium and without fluorogold labelling. Therefore, in order to compare the recovery from inactivation kinetics of fast inactivating Na-currents in large and small neurons under similar conditions, we recorded from unlabelled, control, large DRG neurons in 140 mM extracellular sodium. However, the time constants for recovery from inactivation of unlabelled, large DRG neurons in 140 mM sodium (22.3 ± 4.4 ms at -70 mV, $n = 10$) were similar to those measured in labelled, large cutaneous afferent neurons in reduced extracellular sodium (24.9 ± 4.3 ms at -70 mV, $n = 10$). This indicates that the recovery from inactivation kinetics in large cutaneous afferent neurons is not significantly altered by both fluorogold labelling and reduced extracellular sodium gradients.

We also measured the recovery from inactivation kinetics in axotomized large DRG neurons in 140 mM extracellular sodium. The axotomized neurons in this study were labelled with fluorogold that had been placed in a silicone cuff that was attached to the transected nerve at the time of axotomy. This is the same way that axotomized small DRG neurons were identified by Black et al. (1999). The time constants for recovery from inactivation of these axotomized large DRG neurons ($n = 10$) measured in 140 mM extracellular sodium were similar to those measured in axotomized large cutaneous afferent neurons in reduced extracellular sodium.

In all of the experiments above, the Na-currents were recorded 12–24 h after the DRG neurons were harvested and cultured. However, Liu et al. (2000a) have reported that the excitability of large DRG neurons can change 16–20 h following axotomy. In order to determine if the recovery from inactivation kinetics of large DRG neurons might be altered after 12 h in culture, we also recorded from control and axotomized DRG neurons 2–5 h after dissociation (in 140 mM extracellular sodium). Both control and axotomized large DRG neurons displayed fast recovery from inactivation. At -70 mV the time constant for recovery from inactivation for Na-currents was 15 ± 4 ms ($n = 6$) in large control neurons and 12 ± 3 ms ($n = 6$) in axotomized neurons under these conditions ($P > 0.05$). This indicates that the recovery from inactivation kinetics of large DRG neurons is not significantly altered at larger in the 140 mM extra-cellular sodium, the relative ratio of fast and slow current amplitudes measured in control neurons at 2–5 h after dissociation is similar to that measured after 12–24 h (Table 2). The relative decrease in the fast ($\sim 30\%$) and slow ($\sim 60\%$) Na-current is also similar at the two time points.

DISCUSSION

We have studied Na-currents in control and axotomized large cutaneous afferent A β -type DRG neurons in short-term culture, and compared them to C-type neurons. Control (non-axotomized) A β DRG neurons expressed fast inactivating TTX-S currents with fast recovery from inactivation and slowly inactivating TTX-R Na-current. The fast inactivating TTX-S currents in A β -type DRG neurons are significantly different from the fast inactivating TTX-S currents in small C-type DRG neurons (Cummins and Waxman, 1997) which exhibit much slower recovery from inactivation. Axotomy decreased the amount of slowly inactivating, TTX-R current, but had only relatively minor effects on the fast inactivating TTX-S current. The results presented here, in conjunction with our previous studies (Black et al., 1996; Dib-Hajj et al., 1999, 1996, 1998b) which examined sodium channel mRNA expression in DRG neurons, provide new information into the molecular physiology of injured peripheral sensory neurons, and may have implications for understanding the molecular pathophysiology of peripheral nerve injury.

The TTX-S current in control A β -type neurons exhibits fast repriming kinetics

The TTX-S currents in uninjured A β neurons displayed fast recovery from inactivation. At -80 mV, the A β neurons reprimed with a time constant of ~ 25 ms, whereas small C-type DRG neurons reprimed with a time constant of ~ 150 ms (Black et al., 1999). The faster recovery from inactivation was observed at all voltages tested. The differences in repriming kinetics could have important implications for excitability and repetitive firing properties. Because the TTX-S current in A β -type neurons reprimed much faster than the TTX-S current in small C-type neurons, the A β -type neurons would be expected to sustain higher firing frequencies. Indeed, this has been shown to be the case (Harper and Lawson, 1985). Waddell and Lawson (1990) reported that while A-fibers can typically follow firing frequencies of 100 Hz C-fibers follow significantly lower frequencies of stimulation (~ 16 Hz). This suggests that the repriming rate of sodium channels may be an important determinant of maximum following frequency.

Previously, Cummins et al. (1998) have shown that the human PN1 (Nav1.7) sodium channel isoform, when heterologously expressed in HEK293 cells, has relatively slow recovery from

inactivation kinetics that are similar to those of the TTX-S current in uninjured small C-type neurons. This raises the possibility that the predominant TTX-S sodium channel in the A β neurons is not PN1, but is generated by different sodium channel isoforms with fast repriming kinetics. If so, what isoform might produce the TTX-S current in A β -type neurons? Black et al. (1996) reported that large DRG neurons (> 45 μ m) expressed high levels of transcript for type-I (Nav1.1) and Na6 (Nav1.6) sodium channels, although cutaneous and muscle afferents were not separately characterized. The repriming kinetics of type-I and Na6 channels have not yet been studied in mammalian expression systems; therefore, both of these isoforms are possible candidates for encoding the A β -type TTX-S current with fast recovery from inactivation.

Changes in repriming kinetics following nerve injury could have important pathophysiologic implications. The TTX-S current in small neurons repriming much more rapidly following sciatic nerve transection than the TTX-S current in uninjured C-type neurons (Cummins and Waxman, 1997). Waxman et al. (1994) found that type-III (Nav1.3) sodium channels are upregulated in DRG neurons following axotomy, and Cummins and Waxman (1997) proposed that type-III channels might contribute to the increase in rate of TTX-S Na-current repriming that occurs in small DRG neurons following axotomy. While type-III channels are also upregulated in large DRG neurons following axotomy (Waxman et al., 1994), we did not observe significant differences in the repriming kinetics of A β -type neurons following axotomy. However, the TTX-S Na-currents in both control and axotomized large DRG neurons exhibited rapid repriming, and therefore, the expression of type-III channels might be more difficult to discern.

Several studies have indicated that following nerve injury A-type DRG neurons exhibit enhanced excitability and are more likely to generate spontaneous activity than C-type neurons (Boucher et al., 2000; Liu et al., 2000b,c). We did not see an increase in the amplitude of the TTX-S current following axotomy, as Rizzo et al. (1995) suggested might be the case. Instead we observed a slight decrease in the TTX-S current amplitude. The decrease that we observed in the somatic TTX-S Na-current amplitude following axotomy would not be expected to increase neuronal excitability. One possibility is that the current properties in dissociated DRG neurons are different from those *in vivo*; i.e. the dissociation procedure might alter the Na-current properties. Another possibility is that the Na-currents in control and axotomized DRG neurons might differ in some property that we did not characterize in the present study. For example, while we observed a slight decrease in somatic Na-current amplitude, the Na-current density in the axon might increase following axotomy. Alternatively, the distinct properties of the Na-currents in A β -type neurons might predispose these neurons to hyperexcitability. The repriming kinetics was still ~three–four-fold faster at –70 and –60 mV in axotomized A β -type neurons than in axotomized C-type neurons. This faster repriming of the TTX-S Na-currents in A β -type neurons coupled with the axotomy-induced downregulation of potassium current (Everill and Kocsis, 1999) might contribute to ectopic activity generation following nerve injury.

The TTX-R current amplitude in A β neurons was greatly reduced following axotomy, and this was similar to the reduction of TTX-R current that is observed in small neurons (Cummins and Waxman, 1997). Renganathan et al. (2000) have demonstrated that the TTX-R current in cutaneous A β neurons is generated by the SNS (Nav1.8) sodium channel isoform and Dib-Hajj et al. (1996, 1998a) found that axotomy decreases the level of SNS transcript. Because of the complex changes that occur following axotomy, including a downregulation of multiple potassium currents (Everill and Kocsis, 1997, 2000), it is difficult to predict how this reduction in TTX-R current amplitude might impact excitability.

The tail current in A β -type neurons is not generated by SNS or TTX-S channels

Both the control and axotomized A β -type neurons exhibited large inward tail currents. Because large tail currents were observed in axotomized neurons that did not express measurable slowly inactivating TTX-R Na-currents, it is unlikely that they are dependent on the flow of ions through SNS channels. Consistent with this interpretation, TTX-R tail currents are present in large cutaneous afferent DRG neurons from SNS-null mice (Renganathan et al., 2000). Na channel α -subunit (NaN) channels, the only other TTX-R channel that has been identified in DRG neurons, are not expressed at significant levels in large DRG neurons and are down-regulated following peripheral axotomy (Dib-Hajj et al., 1998b, 1999; Tate et al., 1998). Therefore, it is also unlikely that NaN channels underlie the tail currents observed in A β neurons. Raman et al., (1997) proposed that Na δ channels generate 'resurgent' tail currents in cerebellar Purkinje cell neurons. However, these resurgent currents in Purkinje neurons are TTX-S, and the large tail currents in A β neurons are not reduced by TTX. Therefore, Na δ and other TTX-S sodium channels probably do not contribute significantly to the large tail currents in A β -type neurons. The tail currents might involve an ion other than Na⁺, or could arise from another channel type that is not blocked under our ionic conditions.

CONCLUSION

Our data on A β -type neurons demonstrate that these neurons have Na-currents with different properties from the Na-currents in small DRG neurons, which are predominantly nociceptors. These data, in conjunction with data on mRNA expression levels in large neurons (Black et al., 1996), suggest that the predominant TTX-S sodium channel isoform is different in A β neurons and small DRG neurons. This difference, observed in neurons before and after axotomy, is likely to contribute to the different excitable properties of these populations. TTX-S Na-current properties, in addition to an alteration in SNS and TTX-R currents, could contribute to the hyperexcitability observed in some DRG neurons after axotomy (Liu et al., 2000a). Given that A β -type hyper-excitability has been implicated in neuropathic pain following injury (Gracely et al., 1992), drugs targeted at the sodium channel isoforms preferentially expressed in A β neurons may therefore be appropriate for the treatment of some types of chronic pain.

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Abbreviations

DRG	dorsal root ganglion
EGTA	ethylene glycol-bis(β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
HEPES	<i>N</i> -(2-hydroxyethyl)piperazine- <i>N'</i> -(2-ethanesulfonic acid)
NaN	Na channel α -subunit
SNS	sensory neuron-specific
TMA	

tetramethylammonium chloride

TTX

tetrodotoxin

TTX-R

tetrodotoxin-resistant

TTX-S

tetrodotoxin-sensitive

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Fig. 1.

Differences in fast and slow inactivating currents of large (48–50 μm) cutaneous afferents after axotomy. (A) Typical fast and slow decaying sodium current recorded from a control large afferent DRG neuron. (B) Recording from an axotomized large afferent DRG neuron displaying only fast decaying Na-current typical of this condition. (C) Demonstrates the steady-state voltage inactivation curves for the traces in (A) and (B). The normalized peak current of each individual trace is shown for control (Δ) and axotomized ($\circ$) cells plotted against the prepulse voltage. Note the inflection in the control curve (at ~ -40 mV). This inflection is indicative of two distinct sodium current components. (D) Shows the pulse protocol used to evoke these current traces. Membrane potential was held at -80 mV and a prepulse, ranging from -130 mV to -5 mV in 5 mV steps, was applied for 500 ms. The test pulse was to 0 mV for 20 ms. The peak current elicited by each test pulse is measured for each prepulse voltage.

Fig. 2.

Pharmacological examination of tail currents of large cutaneous afferent DRG neurons. A_i shows a family of currents from an axotomized large cutaneous afferent DRG neuron before presentation of 250 nM TTX. This family of inward currents was initiated by holding the membrane potential at -80 mV and stepping from -130 mV to -5 mV in 5 mV steps for 500 ms, stimulation phase of the pulse was to 0 mV for 20 ms (see Fig. 1). Typically, as with a large proportion of ligated cells, no slow current is visible, only fast activating/inactivating current. The tail currents in this cell are around 5 nA. B_i demonstrates the effects of 250 nM TTX after 3 min; all fast current is seen to disappear, the tail current however is left untouched indicating that it is not generated by TTX-S sodium channels. Confirmation of this lack of effect on the tail current is seen in the two lower panels A_{ii} and B_{ii} , where a further protocol was applied to the same cell. A subtraction of A_{ii} (before TTX) from B_{ii} (after TTX) reveals a simple straight line (data not shown), which indicates no discernible difference between the two recordings. The pulse protocol used to initiate the current traces in A_i and A_{ii} is identical to that of Fig. 1. The pulse protocol for traces B_i and B_{ii} consists of holding the cell at -80 mV prepulsing to $+30$ mV for 50 ms, then stepping from -10 down to -90 mV in 10 mV steps for 50 ms to elicit tail currents as demonstrated in the figure.

Fig. 3.

TTX-S sodium currents in large cutaneous afferent DRG neurons exhibit fast recovery from inactivation. (A) A typical example of current traces obtained from a *large* cutaneous afferent DRG neuron with only fast inactivating current detectable. The ms values indicate recovery duration (Δt); cells, held at -80 mV, were prepulsed to -20 mV for 20 ms to inactivate voltage activated current, then brought back to -80 mV for increasing recovery durations prior to a test pulse of -20 mV. (B) Gives a comparison with a recording taken from a *small* C-type neuron which also has mostly fast inactivating current. (C) Shows a graphical representation comparing the difference in repriming times of the small and large neurons. (D) Demonstrates the pulse protocol used to examine the recovery kinetics. In order to accommodate the long prepulse duration used in the recovery from inactivation protocol, the sample rate was reduced to 5 kHz for this protocol.

Fig. 4.

Graphic representation of recovery from inactivation time constant for TTX-S Na-currents following axotomy in both large, A β -type, and small, C-type, neurons. Top graph (A) shows time constants for recovery from inactivation in TTX-S control and axotomized large (49–50 μ m) neurons. The insert on the top graph (A) shows the pulse protocol used to initiate the currents which are graphically shown in both (A) and (B). The voltage at which the currents were allowed to recover from inactivation (V_{rec}) is plotted on the horizontal axis. The time constant for recovery from inactivation was obtained by fitting a single exponential function to recovery time courses such as those shown in Fig. 3C. Lower graph (B) demonstrates the difference between rates of inactivation of large, TTX-S control ($n = 21$) and axotomized (n

= 12) neurons (broken lines), and small (20–30 μm), control ($n = 15$) and axotomized ($n = 15$) neurons (solid lines; from Black et al., 1999). The error bars indicate mean $\pm$ S.E.M.

Table 1

Solutions for voltage clamp experiments

Solutions	Intracellular	Extracellular	
	I	E1	E2
NaCl ₄	10	20	20
TMACl, mM*	0	115	115
KCl, mM [†]	0	3	3
CsCl ₂ , mM§	125	0	0
CaCl ₂ , mM [†]	0	1	1
MgCl ₂ , mM [‡]	2	1	1
CdCl ₂ , μM [†]	0	100	100
HEPES, mM*	10	10	10
EGTA, mM*	1	0	0
TTX, nM [§]	0	0	250
pH	7.2	7.4	7.4
Mosmol	300–305	305–310	305–310

Table 2

Comparison of mean peak amplitude in control and axotomized large cutaneous afferent DRG neurons

	Experimental condition	Mean peak amplitude $\pm$ S.E.M.
Fast component (TTX-S)	Control ($n = 8$)	27.6 ± 1.92
	Axotomy ($n = 15$)	22.3 ± 1.43
Slow component (TTX-R)	Control ($n = 20$)	6.96 ± 1.05
	Axotomy ($n = 14$)	2.65 ± 0.59