Axotomy Increases the Excitability of Dorsal Root Ganglion Cells With Unmyelinated Axons

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Axotomy increases the excitability of dorsal root ganglion cells with unmyelinated axons. J. Neurophysiol. 78: 2790–2794, 1997. To better understand the neuronal mechanism of neuropathic pain, the effect of axotomy on the excitability of dorsal root ganglion (DRG) cells with unmyelinated axons (C cells) was investigated. Whole cell patch-clamp recordings were performed on intact DRG cells with intact axons or with axons transected 7–12 days earlier. C cells were identified by 1) soma size, 2) action potential morphology, 3) conduction velocity, and 4) in some cases, injection of Fast Blue into the injured nerve fibers. Axotomy reduced (more negative) action potential threshold but did not significantly change resting membrane potential, action potential duration, or maximal depolarization rate. Axotomy significantly increased the peak sodium current measured under voltage-clamp conditions. In Fast Blue–labeled (injured) cells, the tetrodotoxin (TTX)-sensitive current was enhanced while the TTX-resistant current was reduced. These results suggest that axotomy increased the excitability of C cells, possibly because of a preferential increase in expression of TTX-sensitive sodium currents.

INTRODUCTION

The mechanism of neuropathic pain following nerve injury is unresolved, but evidence suggests that somata of the dorsal root ganglion (DRG) may become an important source of abnormal spontaneous activity after an injury of the peripheral nerve (Burchiel 1984; Devor et al. 1994; Wall and Devor 1983; Xie et al. 1995; Zhang et al. 1997). Patch-clamp recordings of the spontaneous activity initiated in the somata of isolated DRG cells after nerve injury are suggestive of enhanced excitability (Petersen et al. 1996; Study and Kral 1996). Previous studies from dissociated DRG cells of the A classification indicate that peripheral axotomy induces a selective reduction in tetrodotoxin-resistant (TTX-R) Na+ currents and an enhancement in tetrodotoxin-sensitive (TTX-S) Na+ currents in dissociated cutaneous afferent neurons, perhaps accounting for the enhanced excitability (Rizzo et al. 1995).

In the present study we hypothesized that axotomy would enhance the excitability of C cells through alterations in Na+ channel characteristics. Little information is presently available on these cells with unmyelinated fibers, although their role in pain perception is well established. Experiments were undertaken in which patch-clamp techniques were applied to cells in the intact ganglion (Fig. 1A). This preparation was developed in our laboratory as an alternative to isolated cell recording. It offers the advantage of maintaining axonal connections, thereby allowing the measurement of conduction velocity while minimizing potential alterations in cellular phenotype due to culture conditions or removal from a normal cellular environment (Zhang et al. 1996).

METHODS

Ligation procedure

Three-day-old Sprague-Dawley rats were deeply anesthetized with ether. The right sciatic nerve was exposed at the midthigh, tightly ligated, and sectioned just distal to the ligation site. In some rats, 1–2 μl Fast Blue dye 1% in normal saline (Sigma Chemical Co.) was injected into the nerve proximal to the ligature to later identify the soma of DRG cells with ligated axons. The distance between the stump and the muscle branch of the nerve at the level of the ilioinguinal ligament was ~5 mm. This distance was later proved to be enough to prevent any dye diffusing within the epineurium from reaching the proximal muscle branches.

In vitro recording

Seven to 12 days after nerve ligation, the rat was deeply anesthetized by an intraperitoneal injection of pentobarbital sodium (0.1 ml, 6 mg/ml). The sciatic nerve was isolated from the surrounding tissue and traced to the ganglion. After the bone covering the spinal cord was removed, L4 and L5 ganglia with nerve and dorsal roots attached were placed in a small petri dish. The epineurium and perineurium that formed the capsule were removed and the dorsal roots were transected adjacent to the ganglia. The remaining tissue was incubated with gentle agitation in Ca2+- and Mg2+-free N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-buffered saline (composition, in mM: 140 NaCl, 3 KCl, 25 glucose, and 10 HEPES, pH 7.3) with 1 mg/ml collagenase (Boehringer type P) for 30 min at 35 ± 1°C. The ganglion with attached nerve was placed in a recording chamber that was mounted on the stage of an inverted microscope. The peripheral nerve was led into a suction electrode for stimulation. The DRG was continuously perfused at 2 ml/min with oxygenated HEPES at room temperature (23–24°C).

Gigaohm seals were achieved with the use of glass microelectrodes with impedances of 2–4 MΩ. Whole cell recordings were performed with the use of the Axopatch-1D amplifier. The external recording medium used when measuring the action potential (AP) threshold was HEPES saline, as specified above, with the addition of 1 mM CaCl2 and 1 mM MgCl2. The pipette solution contained (in mM) 140 KCl, 1 CaCl2, 2 MgCl2, 11 ethyleneglycol-bis-β-aminoethyl-ether-N,N′,N′,N′-tetraacetic acid (EGTA), 2 Mg-ATP, and 10 Na-HEPES, pH adjusted to 7.3 with 1 N KOH. Na+ currents were recorded under conditions of reduced Na+ gradient to reduce the magnitude of the Na+ current and thus improve the space-clamp properties of the recording. The external solution contained (in mM) 30 NaCl, 110 N-methyl-d-glucamine (NMDG), 3 KCl,
INCREASED EXCITABILITY OF AXOTOMIZED C-TYPE DRG CELLS

1 CaCl₂, 1 MgCl₂, 0.1 CdCl₂, and 10 HEPES, pH adjusted to 7.3 with 1 N NaOH. The pipette solution contained (in mM) 110 CsF, 25 NaCl, 1 CaCl₂, 10 EGTA, 20 tetraethylammonium, 10 glucose, and 10 HEPES, pH adjusted to 7.3 with 1 N CsOH. NMDG served as the nonpermeant monovalent cation in place of external Na⁺. Internal Cs⁺ salts and external Cd²⁺ were used to minimize current through voltage-dependent K⁺ channels and Ca²⁺ channels, respectively. Tetrodotoxin (TTX) was initially dissolved in a dilute acetic acid solution to a stock concentration of 100 µM and diluted to final concentration of 1 µM.

After the cell-attached configuration was obtained, the conduction velocity was measured. An orthodromic AP was evoked by applying current pulses (3–5 mA, 2–6 ms, 1 per s) to the peripheral nerve through a nerve suction electrode. The conduction velocity was calculated by dividing the latency of the AP into the distance between the stimulating electrode and the center of the ganglion. Capacity compensation and series resistance compensation (>80%) were used to minimize voltage errors following the start of whole-cell recording. The whole cell capacitance (pF), access resistance, and input resistance were calculated on the basis of the steady-state current changes following a voltage step from −60 to −80 mV. The cell surface area (µm²) was calculated as 100 × whole cell capacitance for each cell. Leak subtraction of the current through the leak conductance was performed on line with the use of a P/4 protocol described by Bezanilla and Armstrong (1977). For measurement of AP threshold, the recording mode was switched to the current-clamp mode and a series of depolarizing current pulses was delivered. To standardize the recording conditions, a hyperpolarizing or depolarizing steady-state current was applied to bring the starting potential to −60 mV. Depolarizing currents over the range of 0.05–2 nA in increments of 0.05 nA (duration 20 ms) were applied until an AP was evoked. The AP threshold was defined as the first point on the rising phase of the spike at which the voltage change exceeded 50 mV/ms. The voltage drop at the pipette tip was calculated as test current × access resistance for each individual cell and subtracted from the measured AP threshold.

Sodium currents were recorded in voltage-clamp mode. The peak sodium current for each individual cell was normalized to the cell surface area. In some experiments, sodium current recording was performed on Fast Blue–labeled cells that were identified under the fluorescence microscope with the use of wideband ultraviolet excitation (emission maximum 430 nm, excitation maximum 380 nm; Fig. 1, B and C).

In general, identification criteria for a C cell were as follows: 1) a cross-sectional area < 500 µm² (or a diameter < 25 µm) (Nagy et al. 1993; Waddell and Lawson 1990), 2) a conduction velocity < 0.8 m/s (Nagy et al. 1993), and 3) a pronounced inflection on both the rising and falling phases of the AP (Fulton 1987). In conditions in which Na⁺ currents were recorded, the reduction in Na⁺ gradient and the blockade of K⁺ currents dramatically altered the shape of the AP. For these experiments, identification of C cells was based solely on morphological criteria.

RESULTS

Current-clamp recording

The mean diameter for the population of uninjured cells we selected for study was 22.8 ± 0.5 µm (n = 30), which was not significantly different from values for the population of axotomized cells (21.9 ± 0.4 µm, n = 24; P > 0.05, t-test). Similarly, the mean conduction velocity for normal cells was 0.49 ± 0.06 m/s (n = 35), not significantly different from values for axotomized cells (0.51 ± 0.05 m/s, n = 32; P > 0.05, t-test).

Axotomy significantly lowered the AP threshold of C cells from −31.1 ± 1.9 mV (n = 18) to −38.2 ± 1.8 mV (n = 13; Table 1). The resting potential was stable during patch clamp recording. An analysis of the resting potentials showed no significant difference between injured (having transected sciatic axons) and uninjured (with previously intact sciatic nerve axons) cells (Table 1). There were no differences in the mean input resistance, AP duration, or maximum depolarization rate between control and injured cells. However, there may have been subsignificant trends. Average AP duration was 18% greater and the maximum depolarization rate was 20% less in the injured group than in the control group.

FIG. 1. Nerve–dorsal root ganglion (DRG) preparation. A small DRG cell was pulled away from the ganglion by the patch-clamp pipette after a recording experiment. B: representative example of a DRG labeled retrogradely after an injection of Fast Blue into the sciatic nerve 7–12 days earlier. Bright spots: labeled cells. Notice that labeled cells, on and beneath the surface, are all visible. C: same ganglion but with a higher magnification. Scale bars: 40 µm.
TABLE 1.  *AP parameters in normal and nerve-injured DRG cells*

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Axotomized</th>
<th>t-Test</th>
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</thead>
<tbody>
<tr>
<td>$V_{m}$, mV</td>
<td>$-59.2 \pm 1.9$ (27)</td>
<td>$-55.6 \pm 2.2$ (18)</td>
<td>NS</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>271.0 ± 38.3 (38)</td>
<td>226.8 ± 34.9 (31)</td>
<td>NS</td>
</tr>
<tr>
<td>AP threshold, mV</td>
<td>$-31.1 \pm 1.9$ (18)</td>
<td>$-38.2 \pm 1.8$ (13)</td>
<td>$P &lt; 0.05$</td>
</tr>
<tr>
<td>AP duration, ms</td>
<td>5.2 ± 0.5 (21)</td>
<td>6.1 ± 0.9 (12)</td>
<td>NS</td>
</tr>
<tr>
<td>$V_{max}$ (d/dt)</td>
<td>108.6 ± 15.9 (12)</td>
<td>82.7 ± 10.1 (11)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SE, with sample size in parentheses. AP, action potential; $V_{m}$, resting membrane potential; NS, not significant; $V_{max}$, maximum depolarization rate.

Voltage-clamp recording

Current-voltage relationships of C cells were elicited by applying depolarizing pulses 40 ms in duration over the voltage range of $-100$ to $+20$ mV in 10-mV increments. Peak amplitudes of sodium currents were normalized to cell surface area and were plotted against command potentials. Cells from axotomized ganglia exhibited increased inward currents between $-50$ and $-10$ mV (Fig. 2). The mean peak amplitude of total sodium current measured at $-30$ mV was $-1.94 \pm 0.22$ pA/µm$^2$ ($n = 17$) for normal cells and $-2.74 \pm 0.35$ pA/µm$^2$ ($n = 21$) for nerve-injured C cells ($P < 0.05$, t-test). However, the reversal potentials for the two groups were not significantly different (Fig. 2).

If only 54% of the $L_4/L_5$ DRG cells have axons in the sciatic nerve, as reported (Devor et al. 1985), then 46% of the C cells we recorded in axotomized rats would have uninjured axons. To better resolve cell identification, cells that underwent axotomy were specifically identified by injection of Fast Blue into the proximal end of the cut nerve. At the time of recording, the somata of these axons were identified by the positive fluorescence for Fast Blue. After the current-voltage profile was obtained in normal media, cells were exposed to 1 µM TTX to separate the TTX-S Na$^+$ currents from the total Na$^+$ currents. The mean peak amplitude of TTX-S current was significantly increased after axotomy from $1.20 \pm 0.23$ (SE) pA/µm$^2$ ($n = 6$) to $2.19 \pm 0.23$ pA/µm$^2$ ($n = 6$; $P < 0.05$, t-test). The TTX-R current was absent in five of six injured cells and considerably reduced in one cell. In contrast, TTX-R currents were present in all eight cells recorded in normal rats ($P = 0.003$, $\chi^2$ test; Fig. 3).

**DISCUSSION**

The present results demonstrate that following nerve injury there is an enhancement of excitability in C cells as evidenced by a reduction in AP threshold, and that one possible explanation for this reduction is an alteration in sodium channel characteristics. The present results support the hypothesis that increased membrane density of sodium channels may contribute to a lower threshold for AP generation in chronically injured nerve (for review see Devor 1994).

Our observation of a reduction in AP threshold in C cells is consistent with the results of Study and Kral (1996), who demonstrated that dissociated DRG cells had lower AP thresholds if the cells were harvested from rats subjected to a loose ligation of the sciatic nerve. The reduction in AP threshold is likely due to changes in the characteristics of the active (voltage-dependent) currents. Resting potential was unaffected by injury in the present study, a result consistent with previous observations (Czeh et al. 1977; Gurzu and Smith 1988; Petersen et al. 1996; Study and Kral 1996). Previous results have also demonstrated that the input resistance of the cell is not altered after injury (Gurzu and Smith 1988), leaving changes in active currents as the best explanation for the postinjury enhancement of excitability. We pursued this line of inquiry by examining changes in whole cell sodium current and sodium current subtypes, because sodium channels play a pivotal role in the generation of APs, and any change in the expression of sodium currents would be expected to alter excitability.

DRG cells normally express both TTX-R and TTX-S sodium currents, but C cells primarily express TTX-R currents (Ogata and Tatebayashi 1992; Roy and Narahashi 1992). The TTX-R current has a higher activation voltage compared with TTX-S currents (right-shifted activation relationship) (Ogata and Tatebayashi 1992; Roy and Narahashi 1992). The present results support the hypothesis that sodium current types are dramatically altered by nerve injury and that the response is different among sodium channel subtypes. The total peak amplitude of sodium current in C cells was significantly increased after axotomy; this was solely due to an increase in the TTX-S current because the TTX-R current was reduced by nerve injury. This change in the balance of TTX-R and TTX-S current may be a general response among different cell types, because large cells that had Aβ-fibers innervating the skin also exhibited enhanced TTX-S sodium currents after axotomy (Rizzo et al. 1995).

There might be a chance that some of the cells where sodium currents were measured have myelinated fibers, because these cells are not evaluated with the use of conduction velocity. However, we believe it is unlikely. Our frequency distribution of conduction velocities suggests a classification...
by conduction velocity into three groups: C, Aδ, and Aβ. Zhang, Song, and LaMotte, (unpublished observations). The cell diameter within the C-type category (<0.8 m/s) was 22.1 ± 0.5 μm (range 15–31 μm). The diameters of cells included in our voltage-clamp experiments were 21.8 ± 0.3 μm (range 17–25 μm), well within the range for cells with C-type conduction velocity.

Because the TTX-S current has a faster and left-shifted activation curve compared with the TTX-R current, it may be expected that this would be manifested in a faster conduction velocity of the C fibers. This was not observed in our data. In fact, no significant change was observed in conduction velocity or whole cell inward current activation. The lack of difference may be due to several factors, including a differential distribution of currents between the soma and axon and (potential) differences in steady-state inactivation that would have altered channel availability to support transmission. Our whole cell current averages may have also contained some cells that were not injured, and this would have moved the average closer to that of uninjured cells. The present data set does not allow a better resolution.

It is possible that axonal Na⁺ channels contribute to the total Na⁺ current measured at the soma; however, we believe that the contribution from the axon is small. Fast, voltage-dependent inward currents were smoothly activating with increasing depolarization and did not evidence space-clamp difficulties. If axonal Na⁺ currents represented a significant portion of the total inward current, then it may be expected that the cable properties along the axon would be manifested in a poor voltage control of current activation; this was not observed.

Our electrophysiological results are generally consistent with the analysis of the effects of nerve injury on cell proteins. The type III sodium channel mRNA (associated with the TTX-S channel) is normally unexpressed in adult neurons, but appears in DRG neurons after axotomy, suggesting a dedifferentiation to a more embryonic phenotype (Waxman et al. 1994). In addition, a significant attenuation of the steady-state levels of transcripts encoding the α-SNS subunit in both small and large DRG cells was observed after axotomy (Dib-Hajj et al. 1996). The α-SNS subunit is associated with a slowly inactivating TTX-R current when expressed in oocytes (Akopian et al. 1996; Sangameswaran et al. 1996). Thus a reduced level of α-SNS following injury may explain the selective loss of TTX-R currents observed in the present study. Increased sodium channel expression was also found at the injury site in axotomized rats (Devor et al. 1993).

It may be expected that a shift from TTX-R to TTX-S current would shorten the AP duration, but this could be negated by a decrease in K⁺ conductances, which has been shown recently for medium-sized DRG cells after sciatic axotomy (Everill and Kocsis 1997). It is possible that the decreased K⁺ conductances might have counterbalanced the narrowed AP duration caused by increased TTX-S current. Also, because the 50% steady-state inactivation voltage of TTX-S current is much more negative than that of TTX-R current (Roy and Narahashi 1992) and C cells expressed predominantly the TTX-S current after axotomy (Cummins and Waxman 1997), the total current may have been inactivated more in axotomized cells than in control cells at AP threshold level. Therefore the maximum depolarization rate may not change even though a shift from TTX-R to TTX-S current was observed in the present study.

Although our rats were relatively young at 10–15 days of age, we believe that the electrophysiological characteristics were mature. Previous electrophysiological studies of DRG cells of developing rats have shown that by 10–15 days of postnatal life, the active and passive membrane properties of C cells are similar to those for adult rats (Fulton 1987). Furthermore, C cells appear anatomically and neurochemically mature by 10–15 days (Fitzgerald and Gibson 1984). Thus it is likely that the changes observed in C cell excitability in young rats after axotomy would occur in adult rats as well.

Some of the DRG cells may have degenerated after sciatic
nerve axotomy. It was found that sciatic nerve axotomy given on the day of birth killed most axotomized neurons (Devor et al. 1985; Yip et al. 1984). However, we believe that our recorded cells survived the axotomy in the present preparation. In previous experiments from our laboratory, we also found that the nerve is grossly atrophied if the axotomy is given on the day of birth. However, we axotomized our rats at 3 days of life and contend that the level of degeneration in C fibers is small on the basis of the appearance of our nerves and ganglia and the documented developmental change in the effects of nerve section. For instance, Devor et al. (1985) report only moderate cell loss if axotomy is performed at 9 days of life. Furthermore, the mortal effects of axotomy occur predominantly in larger cells compared with the small cells recorded in the present study (Bondok and Sansone 1984). Our conduction velocity measurements from all the cells in the current-clamp study also demonstrate that these neurons survived axotomy.

In summary, nerve injury results in an alteration in electrophysiological properties of C cells that makes these cells more excitable. Because nociceptors are a subpopulation of C cells, altered AP thresholds and sodium current expression might contribute to the increased excitability and therefore to the pathogenesis of neuropathic pain after peripheral nerve injury.

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