Upregulation of a Silent Sodium Channel After Peripheral, but not Central, Nerve Injury in DRG Neurons

1Department of Neurology and 2Paralyzed Veterans of America/Eastern Paralyzed Veterans Association Center for Neuroscience Research, Yale University School of Medicine, New Haven, Connecticut 06520; 3Rehabilitation Research Center, Veterans Affairs Connecticut, West Haven, Connecticut 06516; and 4Molecular Pharmacology Unit, Glaxo-Wellcome Research and Development, Medicines Research Centre, Stevenage, Hertfordshire SG1 2NY, United Kingdom

Black, J. A., T. R. Cummins, C. Plumpton, Y. H. Chen, W. Hormuzdiar, J. J. Clare, and S. G. Waxman. Upregulation of a silent sodium channel after peripheral, but not central, nerve injury in DRG neurons. J. Neurophysiol. 82: 2776–2785, 1999. After transection of their axons within the sciatic nerve, DRG neurons become hyperexcitable. Recent studies have demonstrated the emergence of a rapidly repriming tetrodotoxin (TTX)-sensitive sodium current that may account for this hyperexcitability in axotomized small (<27 μm diam) DRG neurons, but its molecular basis has remained unexplained. It has been shown previously that sciatic nerve transection leads to an upregulation of sodium channel III transcripts, which normally are present at very low levels in DRG neurons, in adult rats. We show here that TTX-sensitive currents in small DRG neurons, after transection of their peripheral axonal projections, reprimed more rapidly than those in control neurons throughout a voltage range of −140 to −60 mV, a finding that suggests that these currents are produced by a different sodium channel. After transection of the central axonal projections (dorsal rhizotomy) of these small DRG neurons, in contrast, the repriming kinetics of TTX-sensitive sodium currents remain similar to those of control (uninjured) neurons. We also demonstrate, with two distinct antibodies directed against different regions of the type III sodium channel, that small DRG neurons display increased brain type III immunostaining when studied 7–12 days after transection of their peripheral, but not central, projections. Type III sodium channel immunoreactivity is present within somata and neurites of peripherally axotomized, but not centrally axotomized, neurons studied after <24 h in vitro. Peripherally axotomized DRG neurons in situ also exhibit enhanced type III staining compared with control neurons, including an accumulation of type III sodium channels in the distal portion of the ligated and transected sciatic nerve, but these changes are not seen in centrally axotomized neurons. These observations are consistent with a contribution of type III sodium channels to the rapidly repriming sodium currents observed in peripherally axotomized DRG neurons and suggest that type III channels may at least partially account for the hyperexcitability of these neurons after injury.

INTRODUCTION

It is well-established that after injury to their axons within peripheral nerves, dorsal root ganglion (DRG) neurons and their axons can become hyperexcitable, and this can contribute to neuropathic pain (Devor 1994; Ochoa and Torebjork 1980; Zhang et al. 1997). The postinjury hyperexcitability of small DRG neurons, which give rise predominantly to C-type nociceptive fibers, appears to be due, at least in part, to a change in sodium channel expression because it is accompanied by the emergence of a fast, tetrodotoxin (TTX)-sensitive sodium current characterized by rapid repriming (i.e., rapid recovery from inactivation), together with an attenuation of slow, TTX-resistant sodium currents (Cummins and Waxman 1997). The upregulation of a TTX-sensitive, rapidly repriming sodium current in DRG neurons after axotomy is of special interest because rapid recovery from inactivation is associated with a reduced refractory period that can result in hyperexcitability (Chahine et al. 1994; Yang et al. 1994).

The molecular identity of the channel(s) responsible for the rapidly repriming TTX-sensitive sodium current after axonal injury in DRG neurons has not been established. Immunocytochemical studies with pan-sodium channel antibodies have demonstrated accumulations of sodium channels in injured axonal endings within neuromas (Devor et al. 1989; England et al. 1994, 1996), however, the identity of these channel(s) has not yet established. Although accumulation of SNS/PN3 sodium channels has been reported at injured axonal endings (Novakovic et al. 1998), SNS/PN3 encodes a TTX-resistant channel (Akopian et al. 1996; Sangameswaran et al. 1996), leaving the channel responsible for the increase in rapidly repriming TTX-sensitive current unidentified.

In situ hybridization and RT-PCR both demonstrate an upregulation in the expression of the previously silent type III sodium channel gene, which results in the production of mRNA for type III sodium channels in DRG neurons after axonal injury (Dib-Hajj et al. 1996; Waxman et al. 1994). It has been suggested that the type III sodium channel may provide a molecular basis for the rapidly repriming sodium current in axotomized DRG neurons (Cummins and Waxman 1997). However, the upregulation of type III mRNA expression is not necessarily accompanied by the deployment of type III channel protein because translational regulation and posttranslational modulation, as well as transcriptional regulation, can contribute to the control of ion channel expression within the cell membrane (Black et al. 1998; Hales and Tyndale 1994; Sharma et al. 1993; Sucher et al. 1993). Therefore we have examined the expression of type III sodium channel protein in small DRG neurons after axotomy. In this study, we used patch-clamp recording to study TTX-sensitive sodium currents, together
with immunocytochemical methods with isoform-specific antibodies that are selective for type III sodium channels, to ask whether rapidly repriming current/type III sodium channels are deployed in DRG neurons after injury to their peripheral or central axons and found accelerated repriming, accompanied by increased type III sodium channel immunoreactivity, after peripheral but not central axotomy. We also asked whether the newly deployed type III channels are targeted to a particular part of the injured neurons, and we report that they are present not only within somata but also within distal parts of ganglion cell axons in vitro and in situ, close to the region of transection.

**METH ODS**

In this study, peripheral projections of DRG neurons were axotomized via ligation and transection of the sciatic nerve at the mid-thigh level (Dib-Hajj et al. 1996) or central projections of DRG neurons were axotomized by transection of the dorsal root (dorsal rhizotomy) (Kenney and Kocsis 1997). DRG neurons were studied by patch clamp 7–12 days after peripheral or central axotomy. Immunocytochemical methods also were used 7–12 days after transection of either peripheral or central projections of DRG neurons to study the deployment of brain type III sodium channel protein within these cells. DRG neurons initially were studied after short-term (<24 h) culture; this protocol was chosen to provide as close a match as possible to earlier studies (Cummings and Waxman 1997), which provided quantitative patch-clamp data on the sodium currents in axotomized DRG neurons. Subsequently DRG neurons and their peripheral projections in situ also were studied 7–12 days postaxotomy, using similar immunocytochemical methods.

**Surgery**

For transection of the peripheral projections of the DRG neurons, adult female Sprague-Dawley rats were anesthetized with ketamine/xylazine (40/2.5 mg/kg ip) and the right sciatic nerves were exposed at the midhigh level, ligated with 4-0 silk sutures to prevent regeneration to peripheral targets, transected and the proximal stumps placed in silicon cuffs to prevent regeneration (Waxman et al. 1994). In some animals, hydroxyethylstibamine methanesulfonate (4% w/v; Molecular Probes, Eugene, OR), a retrogradely transported fluorescent label, was placed in the cuff before insertion of the nerve stump. The fluorescent label clearly identified neurons in which their axons were transected. The contralateral sciatic nerves served as controls.

For transection of the central projections of the DRG neurons, adult female Sprague-Dawley rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and a L3 laminec- tomy was performed. An incision was made in the dura, and the L3 and L4 dorsal roots were identified and transected (dorsal rhizotomy) with iridectomy scissors. The lesion was packed with Gel-foam and the overlying muscles and skin were closed in layers with 4-0 silk sutures.

Seven- to 12 days after surgery, rats either were killed with an overdose of ketamine/xylazine and decapitated and the tissue was harvested for cell culture or were anesthetized with ketamine/xylazine, perfused with 4% paraformaldehyde in 0.14 M Sorensen’s phosphate buffer and tissue obtained for immunocytochemical studies.

**Cell culture**

Cultures of DRG neurons were established as described previously (Rizzo et al. 1994). Briefly, axotomized and control (uninjured) lumbar ganglia (L5, L6) were excised, freed from their connective tissue sheaths and incubated sequentially in enzyme solutions containing collagenase and then papain. The tissue was triturated in culture medium containing 1:1 Dulbecco’s modified Eagle medium (DMEM) and Hanks’ F12 medium and 10% fetal calf serum, 1.5 mg/ml trypsin inhibitor, 1.5 mg/ml bovine serum albumin, 10 U/ml penicillin, and 0.1 mg/ml streptomycin and plated on polyornithine/laminin-coated coverslips. The cells were maintained at 37°C in a humidified 95% air-5% CO₂ incubator overnight and then used for patch-clamp investigation or processed for immunocytochemical studies as described previously (Black et al. 1998; Cummings and Waxman 1997).

**Electrophysiology**

Sodium currents in small (18–27 μm diam) DRG neurons were studied after short-term culture (12–24 h). Whole cell patch-clamp recordings were conducted at room temperature (~21°C) using an EPC-9 amplifier and the Pulse program (v 7.89). Fire-polished electrodes (0.8–1.5 MΩ) were fabricated from 1.65-mm Corning 7052 capillary glass using a Sutter P-97 puller. The average access resistance was 2.2 ± 0.6 MΩ (mean ± SD, n = 38) for control cells and 2.1 ± 0.7 MΩ (n = 35) for axotomized cells. Voltage errors were minimized using 70–80% series resistance compensation, and the capacitance artifact was canceled using the computer-controlled circuitry of the patch-clamp amplifier. Linear leak subtraction, based on resistance estimates from four to five hyperpolarizing pulses applied before the depolarizing test potential, was used for all voltage-clamp recordings. Membrane currents were usually filtered at 2.5 kHz and sampled at 10 kHz. The pipette solution contained (in mM) 140 CsF, 1 EGTA, 10 NaCl, and 10 HEPES, pH 7.3. The standard bathing solution was (in mM) 140 NaCl, 3 KCl, 1 MgCl₂, 1 CaCl₂, 0.1 μM CdCl₂, and 10 HEPES, pH 7.3. Cadmium was included to block calcium currents. The osmolarity of all solutions was adjusted to 310 osM.

**Immunocytochemistry**

**ANTIBODIES.** Two anti-brain type III sodium channel polyclonal antibodies were used in these experiments. Initial experiments were performed with antibody K175, which is directed against a polypeptide sequence in the C-terminus region of the sodium channel (see following text); subsequent experiments were performed with anti-type III sodium channel antibody (Alamone Labs, Jerusalem), which is directed against a polypeptide sequence within the intracellular loop joining domains I and II of the sodium channel. Both antibodies yielded similar results.

**ANTIBODY K175 PRODUCTION.** The 21-residue peptide QRLKNIS-SKYDKETIKGRIDC corresponding to amino acid residues 1869–1888+Cys of human brain III was synthesized on a Biosearch 9500 peptide synthesizer using solid-phase Fmoc chemistry. Cleaved peptide was purified by gel filtration and conjugated to a purified protein derivative of tuberculin (PPD) using sulfo-SMCC. Dutch rabbits, presensitized against BCG, were immunized with the resulting conjugate emulsified in incomplete Freund’s adjuvant. The specific antibody response was followed by indirect ELISA using free synthetic peptide as antigen, and the antibody utilized was named K175. The antibody preparations used in this study were affinity-purified using immobilized cognate peptide.

**IMMUNOBLOTTING.** A HEK 293 cell line that stably expresses the human brain type III sodium channel, and a control HEK 293 cell line were grown to confluence at 37°C and 5% CO₂ in Dulbecco’s MOD Eagle medium, containing 10% fetal bovine serum, 1 nonessential amino acids (Life Technologies), and 2 mM L-glutamine. Cells were washed briefly in PBS, then lysed in buffer containing 1% Nonidet-P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, and a proteinase inhibitor cocktail (Boehringer Mannheim). The cell extract was spun at 1,400 g for 10 min at 4°C, the supernatant was recovered and the protein concentration was determined using Pierce BCA reagent before mixing with 2× SDS sample buffer (0.5 M Tris-HCl pH6.8, 10% SDS, 0.2% bromphenol blue, 2% DTT, and 20% glycerol). Eleven micrograms of each sample was loaded on a 6% Tris-glycine precast gel (Novex). Proteins were transferred to nitrocellulose paper using a semidyblotter (Phar-
macia Biotech) for 1.5 h. The blot was incubated for 2 h at room temperature in PBS containing 5% fat-free milk powder, and probed with a human brain type III sodium channel specific antibody (K175) at 4°C overnight in PBS containing 5% fat-free milk powder, 0.1% Tween20. The blot was washed for 45 min in PBS containing 0.1% Tween20 with several changes and incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Sigma) for 1 h at room temperature. It then was washed for 45 min PBS containing 0.1% Tween20. Binding of antibody was binding detected using chemiluminescence with ECL reagent according to manufacture’s instructions (Supersignal, Pierce).

**ANTIBODY CHARACTERIZATION.** Antibody K175 was produced by immunization of a rabbit with a polypeptide specific for human brain type III sodium channel, which is identical to the equivalent rat polypeptide. Alignment of the immunizing type III polypeptide sequence with the equivalent regions of the other sodium channels found in DRG shows that the immunizing sequence is specific for brain type III sodium channels (Table 1). Western blot analysis of antibody K175 showed that it reacted with a predominant species at ~260 kDa (Fig. 1).

**IMMUNOSTAINING.** Coverslips with neurons derived from control or axotomized L3/L4 DRG and maintained in vitro for <24 h were processed for immunocytochemistry as follows: 1) complete saline solution, twice, 1 min each; 2) 4% paraformaldehyde in 0.14 M Sorenson’s phosphate buffer, 10 min; 3) PBS, three times, 3 min each; 4) PBS containing 20% normal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100, 15 min; 5) primary antibody [rabbit K175, 1:100, or anti-type III antibody (Alamone), 1:100, in blocking solution without Triton], overnight at 4°C; 6) PBS, six times, 5 min each; 7) secondary antibody [goat anti-rabbit IgG-Cy3, 1: 3000]; and 8) PBS, six times, 5 min each.

Fourteen-micrometer cryosections of intact control and axotomized DRG and sciatic nerves were mounted on poly-l-lysine coated glass slides and processed for immunocytochemistry as described above with the following minor modifications: slides were incubated in 50 mM NH4Cl (20 min, room temperature) to reduce autofluorescence, the slides were not incubated in 4% paraformaldehyde, and slides were incubated in blocking solution for 30–45 min. After the immunocytochemical procedure, the slides were mounted with Aqua-poly mount and examined with a Leitz Aristoplan light microscope equipped with bright field, Nomarski and epifluorescence optics. Images were captured with a Dage DC-330T color camera and Scion CG-7 color PCI frame grabber. Digitized images were manipulated in Adobe Photoshop, with control and experimental tissue being processed in identical manners.

Control experiments included incubation without primary antibody and preadsorption of the antibody with 100–500 M excess of immunizing peptide. Only background levels of fluorescence were detected in the control experiments.

**TABLE 1. Alignment of sodium channel isoform sequences corresponding to region of immunizing polypeptide for antibody K175**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Type</th>
<th></th>
</tr>
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<tbody>
<tr>
<td>Rat brain III</td>
<td>QRLKNISSKYDKET1KGRID</td>
<td></td>
</tr>
<tr>
<td>Rat brain II</td>
<td>QVKXKVSYY1YKKDKGKEDEG</td>
<td></td>
</tr>
<tr>
<td>Rat brain I</td>
<td>RTVQGASFYTKINK1KGGAN</td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>RQHVSIS1Y1K1GGRDRD-DO</td>
<td></td>
</tr>
<tr>
<td>PN1</td>
<td>LRLKDRS8888SQQVFCNGDL</td>
<td></td>
</tr>
<tr>
<td>SNS</td>
<td>RSLTSLNLTHYPRAEDGVS</td>
<td></td>
</tr>
<tr>
<td>NaG</td>
<td>QSDKKIQDIPEDDPBD</td>
<td></td>
</tr>
</tbody>
</table>

**RESULTS**

**Sodium currents in DRG neurons**

In a previous study, it was reported that the TTX-sensitive current in peripherally axotomized small (18–27 μm diam) DRG neurons recovered from inactivation (reprime) more rapidly than the TTX-sensitive currents in uninjured neurons (Cummins and Waxman 1997). We confirmed this observation in the present study: whereas 86% of peripherally axotomized (identified by fluorescent label backfill) DRG neurons (n = 35) recovered rapidly from inactivation, <20% of control neurons did (n = 36). However, in our previous study, repriming kinetics were only measured at −100 mV. In the present study, we therefore asked whether TTX-sensitive sodium currents in axotomized small neurons also reprime much faster than control neurons at voltages near the expected resting potential for DRG neurons. Figure 2 shows representative results at a holding potential of −80 mV and demonstrates that repriming was more rapid in peripherally axotomized neurons. We examined the time course for repriming at voltages ranging from −140 to −50 mV. Figure 3 shows that the TTX-sensitive currents in the axotomized small neurons reprime more rapidly than the TTX-sensitive current in control neurons throughout this voltage range. These results show that the distinct repriming kinetics for the TTX-sensitive current in peripherally axotomized neurons do not simply reflect a shift in the voltage dependence of recovery from inactivation; rather, they suggest that the predominant TTX-sensitive channels in peripherally axotomized small neurons are fundamentally different from those in uninjured neurons.

We also asked whether transection of the central projections (dorsal roots) of small DRG neurons elicited the emergence of a similar rapidly repriming TTX-sensitive sodium current in these neurons. In contrast to peripherally axotomized neurons, which exhibited a significant shift in repriming kinetics, the repriming kinetics of centrally axotomized and control DRG neurons were identical at −80 mV (Fig. 4; τ50 = 145 ± 15, n = 25, and 147 ± 15, n = 25, respectively). Figure 4 also shows that the repriming kinetics of the TTX-sensitive currents in centrally axotomized neurons was similar at all voltages examined from −140 to −50 mV. These results indicate that transection of the central projection of small DRG neurons does not induce the appearance of a rapidly repriming TTX-sensitive sodium current.
Type III expression in DRG neurons

All experiments used two polyclonal antibodies generated against different regions of the type III sodium channel, which yielded similar results.

CULTURED NEURONS. We first studied DRG neurons that had been maintained overnight in culture to match the conditions used in the patch-clamp studies. Sodium channel type III protein was not detected above background levels in most control (nonaxotomized) small (<27 μm diam) DRG neurons (Fig. 5, a and e). Less than 20% of control neurons displayed type III immunostaining above background, and in most of these, the staining was at a low level. In contrast, most small neurons derived from peripherally transected DRG displayed substantial type III immunoreactivity (Fig. 5, b and f). Preadsorption of the antibodies with their respective immunizing peptides eliminated type III immunostaining in the axotomized neurons (Fig. 5, c and g).

Transection of the sciatic nerve at midthigh level axotomizes ~70% of the neurons in L4/L5 DRG ganglia (Himes and Tessler 1989); thus to unequivocally identify transected neurons, a retrograde fluorescent label was placed in the cuff of some ligated and transected rats. This procedure allowed colocalization of fluorescent backfill label and type III immuno-

FIG. 2. Kinetics of recovery from inactivation for TTX-sensitive currents are faster in peripherally axotomized neurons. A and B: family of TTX-sensitive current traces from control (A) and axotomized (B) dorsal root ganglion (DRG) cells showing the rate of recovery from inactivation at −80 mV. Cells were prepulsed to −20 mV for 20 ms to inactivate all of the current, then brought back to −80 mV for increasing recovery durations before the test pulse to −20 mV. Maximum pulse rate was 0.5 Hz. C: time course for recovery from inactivation at −80 mV for the peak currents shown in A and B. Recovery is much slower for the currents in the control than the axotomized neuron. Single exponential functions fitted to the data gave time constants of 160 ms for the control neuron and 41 ms for the axotomized neuron.

FIG. 3. Time constants for recovery from inactivation for control neurons (●; n = 15) and peripherally axotomized neurons (○; n = 15) are shown, plotted as a function of voltage. Cells were prepulsed to −20 mV for 20 ms to inactivate all of the current, then brought back to the indicated recovery voltage for increasing recovery durations before the test pulse to −20 mV. Time constants were estimated from single exponential fits to time courses measured with this protocol.

FIG. 4. Time constants for recovery from inactivation for control neurons (●; n = 25) and centrally axotomized neurons (○; n = 24) are shown, plotted as a function of voltage. Cells were prepulsed to −20 mV for 20 ms to inactivate all of the current, then brought back to the indicated recovery voltage for increasing recovery durations prior to the test pulse to −20 mV. Time constants were estimated from single exponential fits to time courses measured with this protocol.
reactivity (e.g., Fig. 6). Of 195 small neurons from three separate experiments that were backfilled with the fluorescent label, 153 (78.5%) displayed type III immunoreactivity above background levels (background defined as signal above preadsorbed signal; Type III antibody: 84/99, 85.8%; K175 antibody: 69/96, 71.8%).

Type III immunostaining was not confined to the cell bodies of the peripherally axotomized DRG neurons. In neurons that extended neurites in culture, type III staining was prominent in the neurites and was detectable along their entire length, which in most cases was $\approx 75 \mu m$ (Fig. 7, a and c). This immunostaining tended to be distributed along the entire neurite and did not show pronounced foci of increased immunoreactivity or patchiness suggestive of channel clustering. For quantification, we examined 72 axotomized neurons that displayed neurites and observed type III immunostaining in the neurites of 63 (87.5%) of these cells. Type III immunoreactivity was present within secondary and tertiary neurite branches as well as within the primary neuritic trunk and was also clearly present at the growth cones at the ends of the neurites (Fig. 7, b, and c, inset).

Transsection of the central projection of DRG neurons (dorsal rhizotomy) did not result in increased type III immunoreactivity in most axotomized small neurons (Fig. 5, d and h).

DRG NEURONS IN SITU. Within control DRG in situ, type III immunoreactivity was limited to low-to-moderate levels in $\leq 10\%$ of small DRG neurons (Fig. 8, a and e). In contrast, type III immunostaining was substantially increased in peripherally axotomized small DRG neurons (Fig. 8, b and f). Most axotomized small neurons exhibited at least a moderate level of type III staining, and many of these neurons showed high levels of immunofluorescence. Unlike transection of the sciatic nerve, dorsal root transection was not accompanied by an up-regulation of type III immunostaining in most small DRG neurons (Fig. 8, d and h).

SCIATIC NERVE IN SITU. Control sciatic nerves did not exhibit detectable type III immunostaining at any point along their

![FIG. 5. Brain type III sodium channel immunostaining in control and axotomized DRG neurons in vitro. a and c: control DRG neurons exhibit low or nondetectable levels of type III immunostaining. b and f: somata of peripherally axotomized small DRG neurons display robust type III immunoreactivity. c and g: preadsorption of anti-type III antibodies with peptides eliminates type III immunoreactivity in peripherally axotomized DRG neurons. d and h: type III immunostaining is not present in somata of centrally axotomized small DRG neurons. a–d: antibody K175; e–h: anti-type III antibody (Alamone). Scale bar, 25 \( \mu m \).](http://jn.physiology.org/) Downloaded from http://jn.physiology.org/ by 10.220.33.5 on May 7, 2017

![FIG. 6. Colocalization of type III immunoreactivity and fluorescent retrograde label in peripherally axotomized DRG neurons in vitro. Hydroxystilbamidine fluorescent label was placed in the silastic cuff before placing on the transected sciatic nerve. Ten days postaxotomy the L4 and L5 DRG were cultured and processed for immunocytochemical localization of type III sodium channel. a: type III immunoreactivity (red) is present in some DRG neurons. b: fluorescent backfilled neurons appear white-to-light green. c: neurons in which fluorescent label and type III immunoreactivity are colocalized are yellow (arrow), while backfilled neurons without type III immunostaining are green (arrowhead). Scale bar, 25 \( \mu m \).](http://jn.physiology.org/)

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lengths (Fig. 9c). In contrast, there was marked type III immunoreactivity in the distal region of ligated and transected sciatic nerves; a collar of prominent type III immunostaining was present in the transected axons just proximal to the ligature (Fig. 9, a, b, and e). The region of sciatic nerve constricted by the ligature did not exhibit type III immunostaining nor did the portion of the sciatic nerve proximal to the immunostaining collar (Fig. 9, a and e). Within the region of accumulation of type III immunoreactivity, the distal parts of many individual axons could be observed to exhibit type III immunostaining (Fig. 9b). Preadsorption of the type III specific antibodies with their respective immunizing peptides completely eliminated immunostaining in the ligated sciatic nerve (9, d and f).

DISCUSSION

Although hyperexcitability of DRG neurons has been implicated as an important factor in the pathophysiology of neuropathic pain, its molecular basis has not been elucidated. On the basis of electrophysiological results in axotomized motor neurons (Sernagor et al. 1986; Titmus and Faber 1986) and of observations of increased sodium channel immunoreactivity within axonal endings in neuromas (Devor et al. 1989; England et al. 1994, 1996), postaxotomy hyperexcitability has been attributed, at least in part, to a pathological buildup of sodium channels. Previous studies have been interpreted as suggesting that this is due to abnormal vectorial transport resulting from loss of target membrane within the amputated distal nodes of Ranvier and nerve endings (Devor 1994; Devor et al. 1989; Titmus and Faber 1990). The question, of whether different types of sodium channels might be synthesized in DRG neurons after nerve injury, was raised by Waxman et al. (1994) in a study that demonstrated an upregulation of the previously silent type III sodium channel gene in axotomized DRG neurons. Patch-clamp studies subsequently demonstrated a change in the characteristics of TTX-sensitive sodium currents in DRG neurons after sciatic nerve transection with the emergence of a current displaying a fourfold acceleration of recovery from inactivation that appears to contribute to hyperexcitability (Cummins and Waxman 1997). The data shown in Figs. 2 and 3 extend our previous results by demonstrating that, after peripheral axotomy within the sciatic nerve, the recovery from
inactivation of TTX-sensitive currents is also significantly faster at voltages near the resting membrane potential for DRG neurons (−80 to −60 mV). Axotomized DRG neurons with rapidly repriming TTX-sensitive sodium currents therefore should be able to sustain higher firing frequencies, supporting the suggestion (Cummins and Waxman 1997) that expression of sodium channels with distinct kinetic properties contributes to hyperexcitability in these cells.

When expressed in oocytes, the type III sodium channel is TTX sensitive (Joho et al. 1990; Suzuki et al. 1988). Within the normal nervous system, type III sodium channel mRNA is expressed in embryonic neurons but is strongly downregulated as development proceeds (Beckh et al. 1989; Black et al. 1996; Brysch et al. 1991; Dib-Hajj et al. 1996; Felts et al. 1997). The appearance of type III sodium channel mRNA (Dib-Hajj et al. 1996; Waxman et al. 1994) and protein (this paper) in axotomized DRG neurons therefore was unexpected. We observed similar increases in type III protein in DRG neurons after peripheral axotomy using two different antibodies directed against the type III channel. This increase in type III protein could not have been predicted from the previous demonstration of type III mRNA because increased transcription of channel mRNA not always is accompanied by increased levels of functional proteins (Black et al. 1998; Hales and Tyndale 1994; Sharma et al. 1993; Sucher et al. 1993). The upregulation of the type III sodium channel, moreover, is not part of a global up-regulation of channel synthesis in axotomized DRG neurons because expression of the mRNAs for the TTX-resistant SNS/PN3 (Dib-Hajj et al. 1996) and NaN (Dib-Hajj et al. 1998) sodium channels is significantly reduced after axotomy.

The data presented here support the hypothesis that the type III sodium channel underlies the TTX-sensitive rapidly repriming sodium current in peripherally injured small DRG neurons (Cummins and Waxman 1997). There is a good correspondence between the percentages of axotomized small neurons (identified by fluorescent backfill) that show TTX-sensitive rapid repriming (86%) and those that have type III immunoreactivity (79%). Moreover, patch-clamp and immunocytochemical studies reveal parallel changes in demonstrating a lack of rapid repriming and type III staining in small DRG neurons after dorsal rhizotomy 7–12 days previously (it should be noted that cultured cells were studied after <24 h in vitro, so that any effect of shearing off the cells’ main stem axon was a short-term one). This latter observation demonstrates that axonal injury in itself is not sufficient to upregulate type III sodium channel protein in DRG neurons. The neuronal response is dependent on whether central or peripheral projections are injured. In agreement with our results, previous stud-

**FIG. 8.** Brain type III sodium channel immunoreactivity in control and axotomized DRG neurons in vivo. a and e: type III immunoreactivity is generally not detectable in small neurons within control DRG. b and f: peripherally axotomized DRG neurons exhibit increased type III immunostaining. Most small DRG neurons display moderate-to-intense type III staining. c and g: preadsorption of anti-type III antibodies with peptides eliminates type III immunoreactivity in peripherally axotomized DRG neurons. d and h: type III immunostaining is not present in centrally axotomized small DRG neurons. a–d: antibody K175; e–h: anti-type III antibody (Alamone). Scale bar, 25 μm.
ies have demonstrated pronounced electrophysiological changes in sensory neurons after peripheral axotomy, whereas central axotomy triggered much smaller electrophysiological alterations in these neurons (Gallego et al. 1987; Gurtu and Smith 1988). Likewise, results have been reported for several other molecules, including heat shock protein 27 (Costigan et al. 1998) and c-Jun (Broude et al. 1997; Kenney and Kocsis 1997), which show significantly altered levels after peripheral, but not central axotomy, presumably at least in part due to deprivation from a peripheral source of trophic molecules (see Dib-Hajj et al. 1998).

The present results demonstrate increased levels of type III sodium channel protein not only in cell bodies but also in the neurites of axotomized DRG neurons. The apparent disparity...
between the in situ results (type III expression at severed axon tips ⇒ expression along the proximal axon trunk) and the in vitro results (type III expression along entire neurite) may reflect the shorter length of DRG neuron axons in vitro, which does not permit the development of a proximo-distal gradient of channels. Irrespective of this, our observations of the presence of high levels of type III protein within the tips of DRG axons in vitro and of the highest levels of type III protein within the tips of transected axons in situ are consistent with results showing that axons within neuromas are hyperexcitable and can act as ectopic impulse generators (Burchiel 1984; Meyer et al. 1985; Scadding 1981; Wall and Gutnick 1974a,b). The present findings provide support for the hypothesis (Cummins and Waxman 1997) that the type III channel produces the rapidly repriming TTX-sensitive sodium current that emerges in small DRG neurons after axotomy and suggest that the activation of the previously quiescent type III sodium channel gene contributes to abnormal hyperexcitability of injured sensory neurons and/or their processes.

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