Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6777–6780, September 1990
Neurobiology

Changed distribution of sodium channels along demyelinated axons

(ion channels/neural conduction/myelin/antibodies/doxorubicin)

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Communicated by Theodore H. Bullock, June 22, 1990

ABSTRACT **Voltage-gated sodium channels are largely localized to the nodes of Ranvier in myelinated axons, providing a physiological basis for saltatory conduction. What happens to these channels in demyelinated axons is not known with certainty. Experimentally demyelinated axons were examined by using a well-characterized polyclonal antibody directed against sodium channels. Immunocytochemical and radioimmunoassay data were consistent with the distribution of an increased number of sodium channels along segments of previously internodal axon. These findings affirm the plasticity of sodium channels in demyelinated axolemma and may be relevant to understanding how axons recover conduction after demyelination.**

The sodium channel is a transmembrane protein that mediates the voltage-dependent sodium permeability of electrically excitable membranes. The presence of sodium channels is of obvious importance for the generation and propagation of action potentials along axolemma. In normal myelinated axons sodium channels are largely localized to the nodes of Ranvier. The most recent electrophysiological and biochemical studies demonstrate a sodium channel density of several thousand channels per μm^2 at the nodes of Ranvier compared with a density of <25 per μm^2 in internodal segments (1–5). In contrast to these observations in myelinated axons, little is known regarding the distribution of sodium channels in demyelinated axons. Such information is important because the resumption of axonal conduction appears the basis for recovery in many demyelinating diseases (6–8). The present report describes the use of an antibody directed against sodium channels to study their distribution along peripheral nerve axons. The immunocytochemical and radioimmunoassay data that follow indicate that the distribution of sodium channels changes along experimentally demyelinated axons.

MATERIALS AND METHODS

Preparation of Antibody. Tetrodotoxin is a compound that binds with high affinity and specificity to sodium channels. Using well-established methods, we purified the tetrodotoxin-binding protein (TTXR) of sodium channels from the electric organ of the eel, *Electrophorus electricus* (9). This large polypeptide alone appears to contain the entire sodium channel apparatus in *Electrophorus* (10, 11).

Polyclonal antibodies to TTXR (anti-TTXR) were raised in rabbits, and their specificity was demonstrated by described radioimmunoassay and immunoprecipitation methods (4, 12). Although some of these antibodies are species specific, others recognize sodium channels from various fish (T.E.F., unpublished data; 13). In particular, the antisera strongly interact with sodium channels in peripheral nerves of *Carassius auratus* (goldfish).

Production of Demyelinative Lesions. The posterior lateral line nerve of *C. auratus* was used in all experiments. These nerves were demyelinated by *in vivo* intraneural microinjection of doxorubicin (Adriamycin), a DNA-intercalating agent that causes delayed subacute demyelination by killing Schwann cells (14). For intraneural injection fish were anesthetized with tricaine methanesulfonate (3-aminobenzoic acid ethyl ester methanesulfonate; Sigma). The lateral line nerve was exposed by a surgical incision, and intraneural injection of doxorubicin was made with a microliter syringe fitted with a 30-gauge disposable needle. The entire procedure was performed under an operating microscope. Eighty-nine nerves were injected with 0.38 μg of doxorubicin, a dosage shown to produce profound demyelination with minimal axonal degeneration (14). At various times after injection, these nerves were examined by immunocytochemical or electron microscopic techniques.

Immunocytochemistry. Lateral line nerves were removed from fish deeply anaesthetized with tricaine methanesulfonate. One- to two-cm lengths of nerve were placed in 4% (wt/vol) paraformaldehyde/0.1 M phosphate-buffered saline (PBS) for 30 min at room temperature. The nerve was rinsed in 0.05 M PBS. Under an operating microscope small fascicles of nerve were teased apart by using watchmaker's forceps. Several control (myelinated) fibers were mechanically desheathed of myelin by using fine beading needles and watchmaker's forceps. The nerve fibers were then dried upon a gelatin-coated slide. The slide was then immersed in 4% paraformaldehyde/0.1 M PBS for 15 min to ensure adhesion of nerve to the gelatin coating. After being rinsed in 0.1 M PBS three times, the slide was immersed in 0.1 M PBS/0.3% Triton (Sigma)/10% normal goat serum for a minimum of 1 hr. Nerves were then treated for immunocytochemistry as follows: The slides were incubated a minimum of 24 hr with anti-TTXR (at a dilution of 1:1000), anti-TTXR that was preadsorbed or blocked with TTXR, or normal rabbit serum (all at 4°C and diluted in 0.1 M PBS/0.3% Triton/10% normal goat serum). After being rinsed in 0.1 M PBS three times, the sections were incubated for at least 1 hr at room temperature with fluorescein isothiocyanate-labeled goat anti-rabbit IgG diluted 1:50 in 0.1 M PBS/0.3% Triton/10% normal goat serum. Slides were then rinsed in 0.1 M PBS three times, dried, coverslipped with 0.05 M PBS/glycerine mixture, and viewed under an epifluorescence microscope (Zeiss). Omission of Triton detergent from the immunocytochemical mixtures did not qualitatively alter the pattern of reactivity, although penetration of the antibodies was greatly reduced.

Photomicrographs were taken at the same parameters (i.e., lamp intensity, aperture diameter, and exposure time were constant). The distribution and brightness of fluorescence were quantified using a digital image processing system (Image Version 1.17 from the National Technical Information Service, Springfield, VA).

Histopathological Techniques. Nerves for histopathological study were fixed in 4% glutaraldehyde/5% sucrose/0.1 M cacodylate for 24 hr. Nerves were then cut into small blocks,

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Abbreviation: TTXR, tetrodotoxin-binding protein.

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postfixed with 2% (wt/vol) OsO_4/0.1 M phosphate buffer, dehydrated in a series of graded ethanol, passed through propylene oxide, and embedded in Epon 812. For electron microscopy ultrathin sections were cut, doubly stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

Radioimmunoassay. The pooled nerve preparations were homogenized in 1% Lubrol PX and centrifuged at 45,700 × *g* for 25 min. The supernatants were diluted in 0.1 M sodium phosphate buffer, pH = 7.5/0.1% bovine serum albumin/1% Lubrol PX to a concentration of <10 mg/100 μl. Samples (100 μl per tube) were incubated with anti-TTXR (1:2000 dilution) for 1 hr at room temperature. Twenty microliters of ^{125}I-labeled TTXR (17 fM or 30,000 cpm; specific activity = 1.06 mCi/nM; 1 Ci = 37 GBq) was then added to each tube and incubated another 2 hr at room temperature. Fifty microliters of Tachisorb-R was added and incubated with gentle shaking for 2 hr at room temperature. Samples were washed twice with 0.1 M sodium phosphate buffer/0.1% bovine serum albumin/0.1% Lubrol PX (1.25 ml per tube) and then centrifuged at 3700 × *g* for 5 min. The supernatants were discarded, and the radioactivity of the pellets was determined with a γ counter. Concentrations of sodium channels were calculated with reference to a standard curve determined by using known quantities of highly purified eel native sodium channel (TTXR).

RESULTS

Immunocytochemistry of Normal Nerve. In normal myelinated axons specific immunoreactivity was restricted to the nodes of Ranvier (Fig. 1*A*). Preadsorption of anti-TTXR antibody with saturating amounts of TTXR eliminated this nodal immunoreactivity. Additionally, no specific nodal immunoreactivity was seen when the nerve was incubated with normal rabbit serum instead of anti-TTXR antibody. Some fluorescence was seen over myelinated internodal segments, but this was never as discrete or intense as that seen at nodes of Ranvier. This fluorescence of myelin was nonspecific because it remained even after blocking the anti-TTXR with TTXR. Several axons were mechanically desheathed of myelin so that bare internodal axolemma could be examined. These acutely desheathed axons mostly showed minimal homogeneous background fluorescence. Areas of intense fluorescence in the desheathed axons were

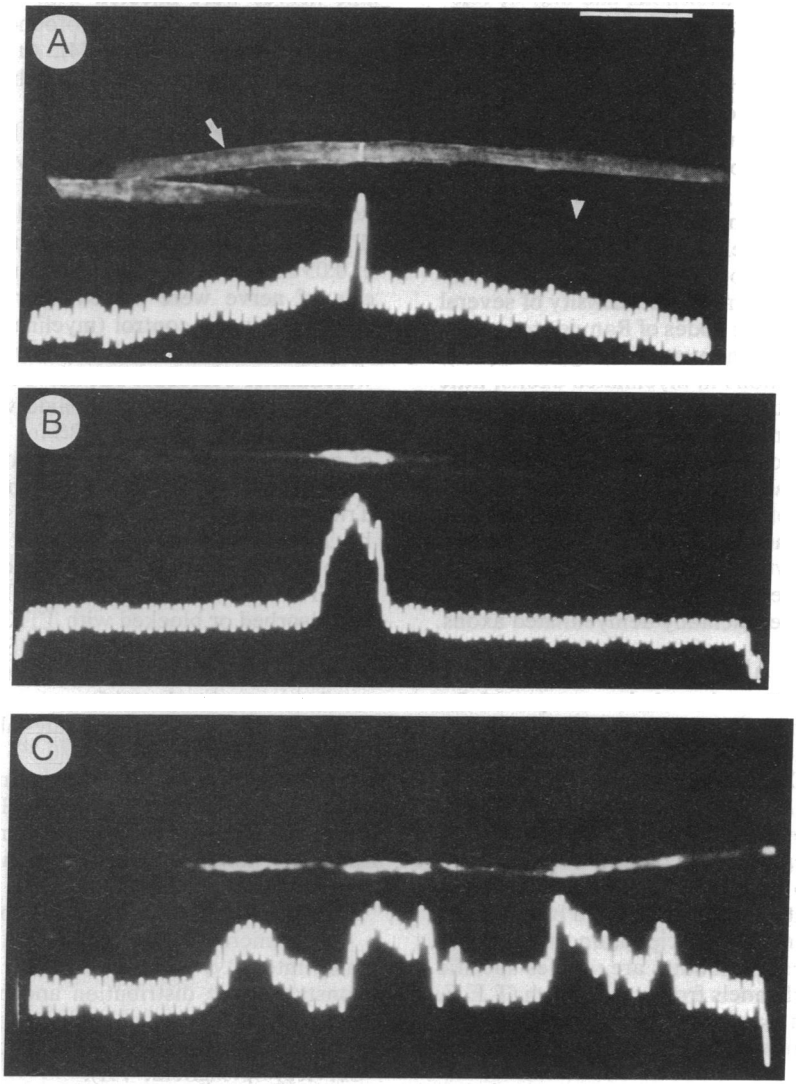

FIG. 1. Sodium channel localization along lateral line nerve axons. In *A*, *B*, and *C* the top image is the fluorescence photomicrograph and the bottom image is the digitized representation showing the relative pixel intensity along the axon length. *(A)* Normal myelinated axon. Note the discrete peak of immunoreactivity at the node of Ranvier, the mild degree of fluorescence of myelinated segments (arrow), and the minimal degree of fluorescence of mechanically desheathed axon (arrowhead). *(B)* Demyelinated axon at 14 days after injection of doxorubicin with one segment of intense immunoreactivity. *(C)* Demyelinated axon at 21 days after injection of doxorubicin with multiple segments of intense immunoreactivity. (Bar = 50 μm.)

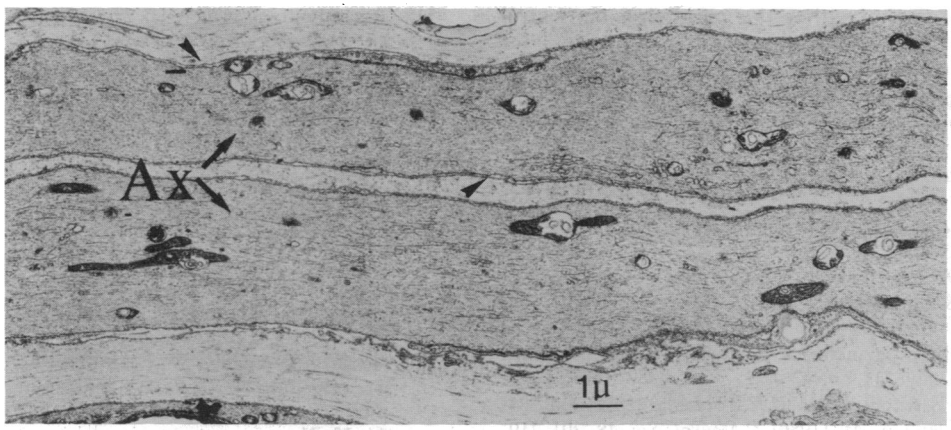

FIG. 2. Electron micrograph of an adjacent section of the nerve shown in Fig. 1*C*. Axons (Ax) are surrounded only by a Schwann cell basal lamina (arrowhead) without Schwann cell cytoplasm or myelin. (Bar = 1 μm.)

seen only in restricted regions corresponding in dimension to nodal axolemma (i.e., ≈1 μm in length).

Immunocytochemistry of Demyelinated Nerve. Thirty of the doxorubicin-injected nerves were examined immunohistochemically with anti-TTXR antibody at various times after injection. As early as 14–21 days after injection specific immunoreactivity occurred over comparatively long segments of demyelinated axons (Fig. 1 *B* and *C*). These segments of immunoreactivity ranged in length from 35 μm to 72 μm—several times longer than nodes of Ranvier. Some demyelinated axons showed multiple regions of specific immunoreactivity, each extending over relatively long stretches of bare axolemma. This kind of immunofluorescence was not observed in either previously adsorbed anti-TTXR antibody preparations or in preparations incubated with normal rabbit serum instead of anti-TTXR antibody.

Electron Microscopic Observations. Correlative electron microscopic studies confirmed that doxorubicin had produced demyelination in these nerves (Fig. 2) just as it does in peripheral nerve of rat (14). Several longitudinal sections of axons from the same nerves and adjacent to the axons examined immunocytochemically showed long segments of demyelinated axons surrounded only by basal lamina.

Radioimmunoassay. The same anti-TTXR antibody was utilized in a radioimmunoassay (RIA) comparing the quantity of sodium channels in control (myelinated) nerves and experimental (demyelinated) nerves. The demyelinated nerves were assayed at day 14 after injection of doxorubicin so that these results could be directly correlated with the immunocytochemical data. Fifty-nine experimental nerves were partitioned into four pooled nerve preparations; 72 control nerves were partitioned into seven pooled nerve preparations. This RIA indicated a highly significant ($P < 0.01$, 2-tailed Student's t test) 4-fold increase in the number of sodium channels per unit of wet weight in the experimental (demyelinated) nerves as compared with the control (myelinated) nerves (mean sodium channel concentration per wet weight of nerve = 18.328 pM per g ± SD of 5.288 for the experimental group vs. 4.473 pM per g ± SD of 1.073 for the control group).

DISCUSSION

These experiments indicate that sodium channels form along the internodal segments of demyelinated axons. Moreover, the intensity and length of the immunoreactivity coupled with the RIA data suggest that channels are being placed *de novo* in these locations. The source of these additional channels is an important question. A sizable body of evidence suggests that some Schwann cells contain voltage-gated sodium channels (15–17), leading to the hypothesis that axonal sodium channels could be locally manufactured within these cells (17). In the present study the appearance of sodium channels occurred along axons without nearby Schwann cells, implying that the channels were synthesized and inserted wholly within neuron–axons. Thus, if Schwann cells are involved in channel turnover or maintenance for axons, they probably provide only an ancillary source. Other recent work (13) using similar immunocytochemical techniques combined with electrophysiological methods has demonstrated that sodium channels accumulate only at the hyperexcitable proximal endings of ligated axons (neuromas). This and other studies (18) suggest that new sodium channel distributions can arise from neuron–axonal influences alone without glial participation.

These kinds of studies are one step in understanding how axons recover function after demyelination. Remyelination and the re-establishment of saltatory conduction are a major means by which conduction can be restored. Discrete foci of inward membrane current, presumably representing future nodes of Ranvier, have been found to precede remyelination in axons demyelinated with lysophosphatidyl choline (19). This observation, by itself, would seem to indicate the formation of new aggregates of sodium channels in demyelinated axons. When remyelination occurs, internodal distances are shorter, indicating the formation of nodes of Ranvier in previously internodal axolemma. In addition, pharmacological studies in remyelinated peripheral nerve have shown an increase in saxitoxin binding that is proportional to this increase in nodal area (20); these results provide evidence that recently formed nodes along remyelinated axons have a relatively normal density of sodium channels, presumably reflecting channel synthesis and insertion after demyelination.

Another possible mechanism of axonal recovery after demyelination is continuous conduction. This mechanism could be particularly important in recovery from demyelination in the central nervous system (e.g., in multiple sclerosis), where limited remyelination occurs (21). Bostock and Sears (22, 23) demonstrated continuous conduction along short segments of axons previously demyelinated by diphtheria toxin, again suggesting a reorganization of sodium channels.

Our findings provide direct morphological and immunological characterization of sodium channel changes in demyelinated axons. However, whether the remodeled sodium channels seen in our study support continuous conduction or serve simply as a prelude to the formation of new nodes of Ranvier is not yet known. Extension of these studies should help us understand how axons can recover function after demyelinative insults. This area of inquiry is particularly important because the primary effect of several human dis-

eases is demyelination of either the peripheral or central nervous system.

This work was supported by National Institutes of Health Grants NS15879 (S.R.L.) and DC00244 (T.E.F.). J.D.E. is supported by a Clinical Investigator Development Award from the National Institute of Neurological Disorders and Stroke (NS01272-03).

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