J. Membrane Biol. 92, 47–56 (1986)

The Journal of
Membrane Biology

Detection of Sodium Channel Distribution in Rat Sciatic Nerve Following Lysophosphatidylcholine-Induced Demyelination

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Summary. In vivo application of lysophosphatidylcholine (LPC) to rat sciatic nerve induces impaired hind leg movement within 2 days which is recovered by 6 days. Segmental demyelination was seen at 2 days after LPC application, and remyelination had barely started in a few axons by 6 days. Using sodium channel-specific monoclonal antibodies and immunofluorescence microscopy, we observed altered distribution of sodium channels in demyelinated axons. Bright fluorescent labeling was found along the segmentally demyelinated axolemma at 6 days in contrast to the dim staining of the demyelinated nerve found at 2 days. In addition, radioimmunoassays detected an elevated number of antibody binding sites on sciatic nerve trunk from the sixth day. Our data provide the immunocytochemical evidence for the assumption that recruitment of sodium channels into demyelinated axolemma contributes to the recovery of function following axon demyelination by LPC.

Key Words sodium channels · monoclonal antibodies · sciatic nerve · demyelination · action potential · lysophosphatidylcholine

Introduction

In normally myelinated axons excitation is confined to the nodes of Ranvier [15]. The internodal membrane currents of myelinated axons are adequately explained on the assumption that the internode is a pure passive cable, with values of resistance and capacitance determined by the myelin [3, 15]. The latter prevents the internodal membrane from being depolarized even if it was excitable.

Demyelination exposes the internodal membrane, and impulse conduction through the bare axolemma is immediately blocked. Such conduction block through demyelinated axolemma was explained on the basis of a change in cable properties [14] between adjacent myelinated and demyelinated regions of the injured axons [3, 16, 34, 40]. In addition, the number of sodium channels at the internodal axolemma seemed too small to sustain conduction when the myelin is lifted [26, 30, 37]. However, with time, conduction can recover [3, 9, 25]. In many cases the recovery of conduction is found before the onset of remyelination, and this recovery was attributed to reorganization of sodium channel as inferred from physiological experiments [3, 18, 25, 35]. Moreover, studies with ^{3}H-saxitoxin and ^{3}H-tetrodotoxin have shown increased numbers of channels in demyelinated axons [27, 28]. Furthermore, morphological evidence suggestive of sodium channel reorganization at demyelinated axons was also obtained using ferric ion ferrocyanide (Fe FeCN) staining [2, 8, 12, 24, 41]. Finally, a change of particles (suggested to be sodium channels) [31, 32, 42] was found following axonal demyelination [38, 40].

This indirect accumulative evidence based on morphological, biochemical and physiological experiments can now be examined directly using sodium channel-specific monoclonal antibodies (mAb's) and immunocytochemistry. The latter enables one to detect the distribution of sodium channels in demyelinated axons. Recently we have generated and characterized two sodium channel-specific mAb's. The mAb SC-72-14 [21, 20] blocks impulse condition by interacting with the so-called "inactivation gate" of the sodium channels, whereas mAb SC-72-38 [1] primarily interacts with the "activation gate" of the channels. The antigenic determinants of these two mAb's are at the external surface of the membrane. The employment of these mAb's in the past, enabled the labeling of sodium channels in the nodes of Ranvier of rat sciatic nerves [1, 19–22].

In this study we used these mAb's to detect sodium channel distribution in axons demyelinated by lysophosphatidylcholine [LPC, 3, 13, 35]. Positive labeling was taken as an indication for the distribution of the antigenic determinants associated with sodium channel activity in demyelinated axon. The efficiency of LPC treatment in causing demy-

elination and in blocking conduction in rat sciatic nerve was measured histologically and by electrophysiological recording across the lesion.

Materials and Methods

Application of Lysophosphatidylcholine (LPC)

Lysophosphatidylcholine (from egg yolk, Sigma L-4129) was dissolved in dimethylsulfoxide (DMSO) to a stock solution of 25 mg/ml which was kept at −20°C. Charles River rats (200-300 g) were lightly anesthetized by ether and their right sciatic nerves exposed. A volume of 20 μl of 0.025% LPC dissolved in saline (LPC treated) or 0.025% DMSO in saline (control) was injected in vivo with a Hamilton syringe into the right sciatic nerve (1.5 cm from the spinal cord). The skin was then sutured back, the animals were revived and each was transferred to a separate cage. Walking ability was tested twice a day until the experiment was concluded.

Measurements of Compound Action Potential (CAP)

LPC-treated and control rats were anesthetized by i.p. injection of pentobarbitone (50 mg/kg body wt). Their two sciatic nerves were dissected separately. Each nerve was placed in Ringer's solution (in mM: NaCl 154, KCl 5.6, $CaCl_2$ 1.8, $MgCl_2$ 0.9, and Tris 10; pH = 7.4) and given a code. Each nerve was taken separately for electrophysiological measurements conducted by someone who was unaware of the treatment that the coded nerve had received.

For measuring the compound action potentials (CAP), each sciatic nerve was aligned on a Lucite chamber. This chamber consisted of a covered narrow slit, the width and depth of which was very close to that of the nerve trunk, such that the volume of short-circuiting external medium was minimal and constant during the perfusion with different external solutions. Nerves were stimulated at 1 Hz by means of a pair of silver/silver-chloride electrodes placed 1 cm distal from the site of LPC injection. The propagated action potential was recorded across the lesion by another pair of electrodes placed at 1 cm proximal to the site of LPC injection. The recording electrodes were connected to a differential amplifier. The distance between the two pairs was 2 cm, and the distance between the electrodes in each pair was 5 mm. The action potential was digitally stored in memory and plotted. All measurements were made at room temperature. The results presented here refer only to the larger and faster wave of the compound action potential which accounts for the larger and faster fibers which could also be examined histologically and immunofluorescently as described below. Note that the recording distance at 1 cm from the site of LPC injection consisted of several space constants (i.e., records were electrically far from the site of LPC injection).

Preparation of Monoclonal Antibodies

As previously described [1, 19–22], we generated sodium channel-specific mAb's using eel electroplax membrane fragments as antigen. Antibodies which specifically modified sodium channel conductance were selected. The supernatant of cloned hybridoma cultures was collected, precipitated by 40% ammonium sulfate, dialyzed extensively against excess Ringer's solution, and concentrated by Amicon to a stock solution of 0.1–1 mg/ml. Culture medium treated as above was used for background subtraction. The amount of IgG was determined by ELISA.

Histological Procedures

Following electrophysiological measurements, 3-mm segments of the sciatic nerve were taken: (i) from the site of LPC injection (i.e., 1.5 cm from the spinal cord; (ii) 1.0 cm distal and (iii) 1.0 cm proximal to the site of LPC injection. These were fixed in 10% formaldehyde, washed in saline, dehydrated by ascending series of alcohols, cleared with chloroform, and embedded in paraffin. Longitudinal and some cross-sections (5–7 μm) were stained by hematoxylene-eosin or by Luxol fast blue (myelin stain). In parallel, single axons were isolated from bundles of the sciatic nerve after fixation. Axons of approximately 20 μm diameter were studied by phase microscopy. The percent of demyelinated axons in each group and the length of the demyelinated region of each axon were measured.

Immunofluorescence Studies

After electrophysiological measurements of impulse propagation, sciatic nerves were transferred into PBS (in mM: NaCl 154, K_2HPO_4 10, $CaCl_2$ 0.9, $MgCl_2$ 0.5, Glucose 10, at pH 7.4). Single axons were separated from 3-mm segments (as above), and in a moist atmosphere the axons were successively incubated at room temperature with: (i) PBS-R (10% rabbit serum in PBS—30 min), (ii) mAb in PBS-R (50 ng/ml) or PBS-R alone (background) for 1 hr, (iii) 3% paraformaldehyde (PF) in PBS (10 min), (iv) rhodamine-labeled rabbit anti-mouse IgG (50 ng/ml, 45 min), (v) 3% PF, (vi) 90% glycerol in PBS. Extensive wash with PBS-R was performed between steps.

Specificity was determined using mAb's SC-72-14 and SC-72-38 pre-absorbed by either a second antibody or by a number of sciatic nerve trunks until the mAb's lost their ability to block the compound action potential. As control we used the eel specific anti-sodium channel mAb ($5D_{10}$, a generous gift of Dr. J. Brockes, MRC, London), which does not recognize the channel in rat sciatic nerve [19, 20].

Axons were photographed at 63 × 10 magnification with a Zeiss fluorescence microscope. Exposures to fluorescent light for 30 sec were followed by standard developing and printing. Staining intensity was measured from photographs illuminated from below with a 100-Watt lamp using a linear photodiode (Pin-10 DP) and a 101A amplifier connected to an electrical recorder. The photodiode sampled the cross section of the axon profile at 1-μm intervals. In the scale for fluorescence intensity, the base line was obtained from a photograph standardly printed from a film not exposed to any light but developed as above, while the top line was obtained from another photograph standardly printed from a film exposed to daylight for 30 sec, then developed as above.

Radioimmunoassay

Sciatic nerves of control and LPC-treated animals were isolated into PBS, pooled into groups, and the nerves of each group were

Table 1. Electrophysiological analysis

Treatment parameter		Naive ($N = 7$)	LPC (2 Days) ($N = 6$)	Control (2 Days) ($N = 6$)	LPC (6 Days) ($N = 6$)	Control (6 Days) ($N = 6$)
APA (mV)	*i*	9.6 ± 3.0	1.07 ± 0.5 ↓ [a]	7.6 ± 1.2	5.6 ± 2.4	8.2 ± 2.2
	c		8.46 ± 3.2	9.2 ± 1.2	7.6 ± 4.2	9.8 ± 1.8
APD_{90}(msec)	*i*	1.15 ± 0.2	1.6 ± 0.4	1.2 ± 0.2	1.88 ± 0.3 ↑ [a]	1.1 ± 0.1
	c		0.98 ± 0.2	1.15 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
CV (m/sec)	*i*	32 ± 5	16.3 ± 1.2 ↓ [a]	28.0 ± 5.0	18.4 ± 4.2 ↓ [a]	29.0 ± 2.0
	c		34.0 ± 3.0	34.0 ± 3.0	32.0 ± 7.0	33.0 ± 1.0
Ir_f(mA)	*i*	0.19 ± 0.07	1.0 ± 0.1 ↑ [a]	0.30 ± 0.05	0.70 ± 0.10	NM
	c		0.2 ± 0.05	0.20 ± 0.05	0.20 ± 0.04	
$T_{Chronaxie}$(μsec)	*i*	110 ± 35	235 ± 50 ↑ [a]	150 ± 35	162 ± 14	NM
	c		120 ± 35	115 ± 43	113 ± 25	

Impulse conduction after LPC injection. Compound action potential was analyzed by the following parameters: (i) the amplitude of the compound action potential (APA) measured from the base line to the maximum amplitude at super-maximal stimulation. (ii) Action potential duration at 90% (APD_{90}) of the wave length measured at super-maximal stimulation. (iii) Conduction velocity (CV) measured from the stimulus artifact to the onset of the impulse. The minimal current required at infinite stimulation time (rheobase) was determined for lowest threshold axons (Ir_f) from the strength duration curve. Stimulus duration required at $2 \times Ir_f$ to bring fast axons to threshold ($\tau_{Chronaxie}$) was measured from the strength duration curve. All measurements were made at room temperature. Stimulation was applied 1 cm distal from the site of LPC injection, and measurements were taken 1 cm proximal to the site of LPC injection. NM—not measured. For statistical analysis, the value of the LPC-treated nerves were compared to the appropriate control nerves at a given time.

[a] $P < 0.002$ at two-tailed *t*-test. Values of contralateral nerves of both LPC and control-injected nerves are also shown. ↑↓ —The arrows indicate increase or decrease of any value.

aligned on a scale, frozen in dry ice and dissected to 3-mm segments. Segments were placed in polystyrene tubes with 100 μml PBS. These were homogenized in a glass-Teflon homongenizer, centrifuged for 10 min at $1000 \times g$. The supernatant was collected, and the membrane fraction was precipitated at $40{,}000 \times g$. The pellet was resuspended in 0.1 ml PBS and sequentially incubated at room temperature (RT) with the following: (i) PBS-R with 0.05% bovine serum albumen (S-PBS) for 1 hr, (ii) mAb's in S-PBS or S-PBS alone (background) for overnight, (iii) ^{125}I-rabbit anti-mouse IgG (50,000 cpm/tube) for 45 min. Extensive wash in PBS was performed between steps until the last wash, which was done with PBS-Tween (0.05% Tween-20 in PBS). Values ($\bar{X} \pm$ SD) after background subtraction are presented. A standard curve with known amount of mAb's (Bio-Yeda) was used to adjust dpm's to femtomoles of bound mAb's. Membrane proteins were determined by the Lowry procedure [17].

Results

Recovery of Impulse Conduction after LPC-Induced Paralysis

Immediately after in vivo injection of LPC the toes of the hind leg, ipsilateral to the injected sciatic nerve, became paralyzed. Disturbance in walking became more severe during the first two days as the animals avoided using their injured leg. Subsequently, a recovery process commenced during which, the paralyzed toes regained their mobility. Abnormal walking was noted, however, up to two weeks after LPC injections. Control animals injected with 0.025% DMSO developed neither paralysis nor walking difficulties.

Impaired walking was accompanied by a significant depression of the action potential amplitude (APA) across the injured region of the sciatic nerve. This APA attenuation is seen two days after LPC injections, but is absent at control-injected nerves (Tables 1). The contralateral sciatic nerves were not affected, implying a local and not a systemic effect. Six days after treatment, a significant increase in the APA was found. This recovery corresponded in time to the improved walking ability evident at six days. Conduction velocity (CV) was 50% slower than normal two days after LPC injection and remained slow at six days despite the increase in the APA (Table 1). We assume that this indicates recovery of impulse conduction in fibers with subnormal conduction velocity. The slow velocity was concomitant with a higher action potential threshold [16]. This was evident by an increase in the minimal current intensity required at infinite stimulus duration (i.e., the rheobase-Ir_f—Table 1) to activate both the fastest (Table 1) and slower (*not shown*) conducting fibers. Stimulus duration at the intensity of twice the rheobase (i.e., the $\tau_{Chronaxie}$) was significantly prolonged at six days (Table 1). Action potential duration at 90% amplitude (Table 1) and 50% duration (*not shown*) was larger six days

Table 2. Histological analysis

Treatment		Nt	Nd	Nd_{1-20}	Nd_{21-40}	Nd_{41-60}	$D_{(\mu m)}$
Naive		250	27	27	—	—	20 ± 7
LPC (2 Days)	*i*	250	208	5	76	127	28 ± 9
	c	250	32	30	2	—	21 ± 3
LPC (6 Days)	*i*	250	140	9	21	110	26 ± 7
	c	250	50	47	3	—	23 ± 2
Control (6 Days)	*i*	250	87	67	20	—	22 ± 8
	c	200	34	32	2	—	21 ± 5

Histological analysis. Demyelination of single axons was measured by phase microscopy. In each experimental group we examined 5 nerves and 50 axons from each. Both contra-lateral (*c*) and ipsi-lateral nerve (*i*) were examined. Measurements were taken from axons along 0.5 cm length from both sides of LPC injection. Nt—total number of examined axons, Nd—the number of demyelinated axons. These are subdivided into Nd_{2-20}, Nd_{21-40}, and Nd_{41-60}, where the length of the demyelinated membrane is denoted by a subscript. Mainly, the largest axons were samples. D—axon diameters measured from cross sections made at the site of LPC or control injection or at 1.5 cm from the spinal cord for nontreated nerves.

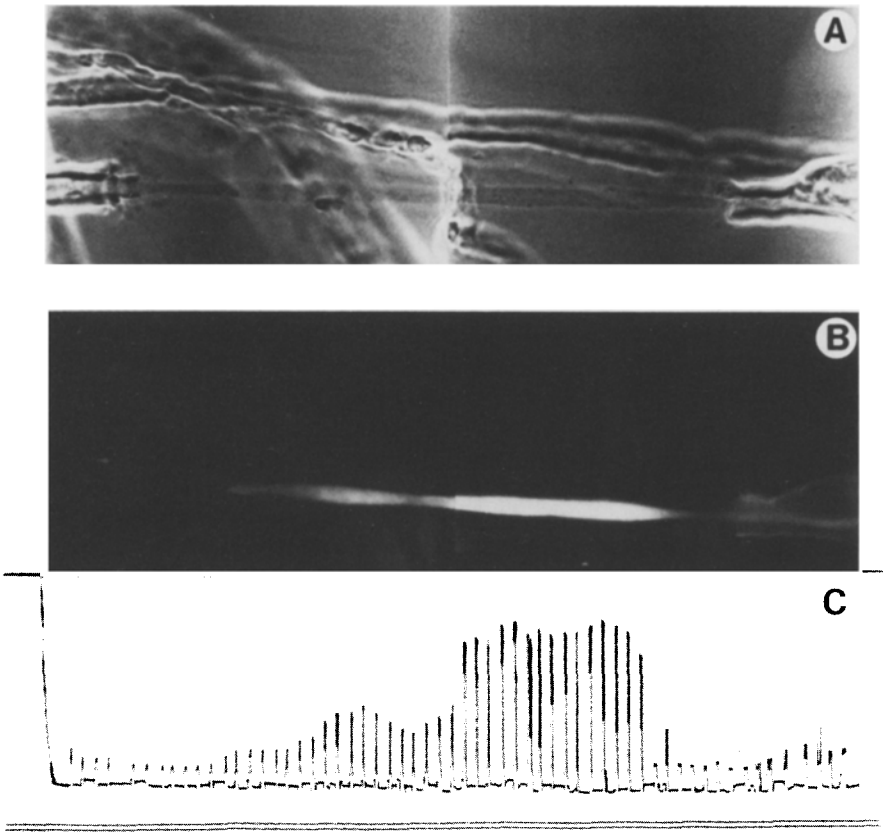

Fig. 1. Immunofluorescence labeling of a severely demyelinated axon obtained from LPC-injected sciatic nerve after six days. (*A*) Phase micrograph showing the bare axolemma of the lower axon beginning to remyelinate at the left side. The other two axons were remyelinated by a very thin layer. (*B*) Fluorescence image. The bare axolemma was unevenly labeled along almost the entire length, whereas the axons with the thin myelin layer were not labeled. To the right, labeling is seen on the outer layer of the myelin and axonal labeling below the myelin. (*C*) Light intensity. Nonuniform light intensity along the demyelinated axon was measured from the picture illuminated from below with a 100-Watt lamp. The linear photodiode sampled the cross section of the axon profile at 1-μm intervals. Measurements were made by an electrical recorder connected to the photodiode

after LPC injection compared to both contralateral and control nerves (Table 1), suggesting that both fast and slow axons were affected.

In summary, recovery of function in LPC-treated nerves appeared in correlation with a threshold increase and a larger time constant in slow conducting fibers. Both the time course and nature of changes were similar to that previously reported [35, 36].

Note that all measurements were made at 1 cm distance from the site of LPC injection (i.e., 10 space constants or more away). At the actual site

of injection action potential configuration was usually peculiar and too difficult for simple analysis.

Histological Analysis

Histological analysis revealed that two days after LPC injection, most axons at the site of the injection (Table 2) and up to 1 cm from both sides (*not shown*) were demyelinated along various lengths. The larger demyelinating length was 60 μm. Fewer axons were less severely affected along 20–40 μm length (Table 2). A very small number of axons remained unaffected (Table 2). The pattern of demyelination was segmental (Figs. 1*A* and 2*B–D*) with several regions of bare axolemma found along the same axon (*not shown*). Considering the space constant of these fibers is 0.5–2 mm [38], such demyelinated length can account for 0.5 space constant or less. At six days after LPC injection, remyelination had just begun, although the APA had significantly increased (*compare* Table 1 to 2). At this time, a few axons were remyelinated by a very thin myelin layer (Fig. 1*A*). The majority of the axons, however, were demyelinated along a length greater than 20 μm (Table 2). Control nerves, injected with the vehicle only, were affected to a much lower degree. In these nerves only a few axons were affected and their demyelination was less severe (1–2 μm, Table 2). Axons contralateral to LPC injections showed no demyelinating profiles (Table 2).

Consistent with the histological findings, conduction deficiencies observed at two days after LPC treatment could be attributed to severe demyelination. However, the recovery of function at six days occurred when only very few axons had started to remyelinate. Thus, remyelination cannot account for the recovery of slowly conducted action potentials in the still demyelinated axons. No significant change in axon diameter was found following LPC injection (Table 2). It is therefore unlikely to assume that such change [23] could account for the recovery of function. No electron microscope (EM) studies were performed. Thus, we cannot say much about a contribution of "improved sealing" between the remaining myelin and the axolemma to the recovery of impulse conduction. However, as previously inferred from other studies [2, 3, 25, 35], recovery of impulse propagation in demyelinated axons could be associated with recruitment of sodium channels by the bare axolemma and these channels may sustain impulse conduction. This was tested by labeling axons with our two sodium channel specific monoclonal antibodies (mAb's).

Table 3. Immunofluorescence labeling

Treatment		*Nt*	*Nd*	*Nds*	Nds_e	Nds_f
Naive		125	15	7	—	7
LPC (2 Days)	*i*	124	107	17	2	15
	c	125	19	13	5	8
LPC (6 Days)	*i*	125	109	95	50	44
	c	125	18	12	4	8
Control (6 Days)	*i*	125	31	27	13	14
	c	125	27	23	17	6

Immunofluorescence labeling. Single axons from rat sciatic nerve were examined at phase and fluorescence light for channel localization. Five ipsi-lateral (*i*) and contra-lateral (*c*) sciatic nerves of each group (25 axons in each nerve) were examined. *Nt*—total number of examined axons. *Nd*—number of demyelinated axons. *Nds*—number of demyelinated axon which were labeled. The latter are subdivided into two groups: Nds_e—non-uniform labeling intensity was seen along the entire demyelinated axolemma. Nds_f—focal recurrent regions (1–2 μm) were labeled along the bare axolemma.

Enlarged Sodium Channel Distribution Along the Injured Axons

In control myelinated axons immunofluorescence staining with both Na-channel specific mAb's was confined to the nodes of Ranvier (Fig. 2*A*). Two days after LPC injection the demyelinated axolemma in the internodal regions was only dimly labeled (Fig. 2*B*). In contrast, six days after LPC injection the demyelinated bare axolemma was brightly labeled (Figs. 1*B* and 2*C*).

Labeling patterns with both Na channel-specific mAb's was similar, implying that the two different sites associated with the two different "gates" of the channel [1, 20] are present along demyelinated axons. In control-injected nerves only a few internodal zones of bare axolemma were labeled in accordance with the small number of demyelinated axons found there (Table 3).

The analysis of axonal labeling found six days after LPC treatment showed the labeled surface area in demyelinated region to be 50–100 times larger than that of a single node. Staining intensity of labeled axons was not uniform (Figs. 1*B* and 2*B* and *C*), and labeled and nonlabeled areas could be frequently seen along the same demyelinated axon. The labeled axons could be subdivided into three subpopulations (Table 3): (i) Axons with nonuniform staining intensity which covered almost the entire demyelinated axolemma (main population—Figs. 1*B* and 2*C*). (ii) Axons where along the bare axolemma we found several labeled foci (1–2 μm length) separated from each other by 10–15 μm (smaller population—*not shown*). The distance between those labeled regions make it unlikely to as-

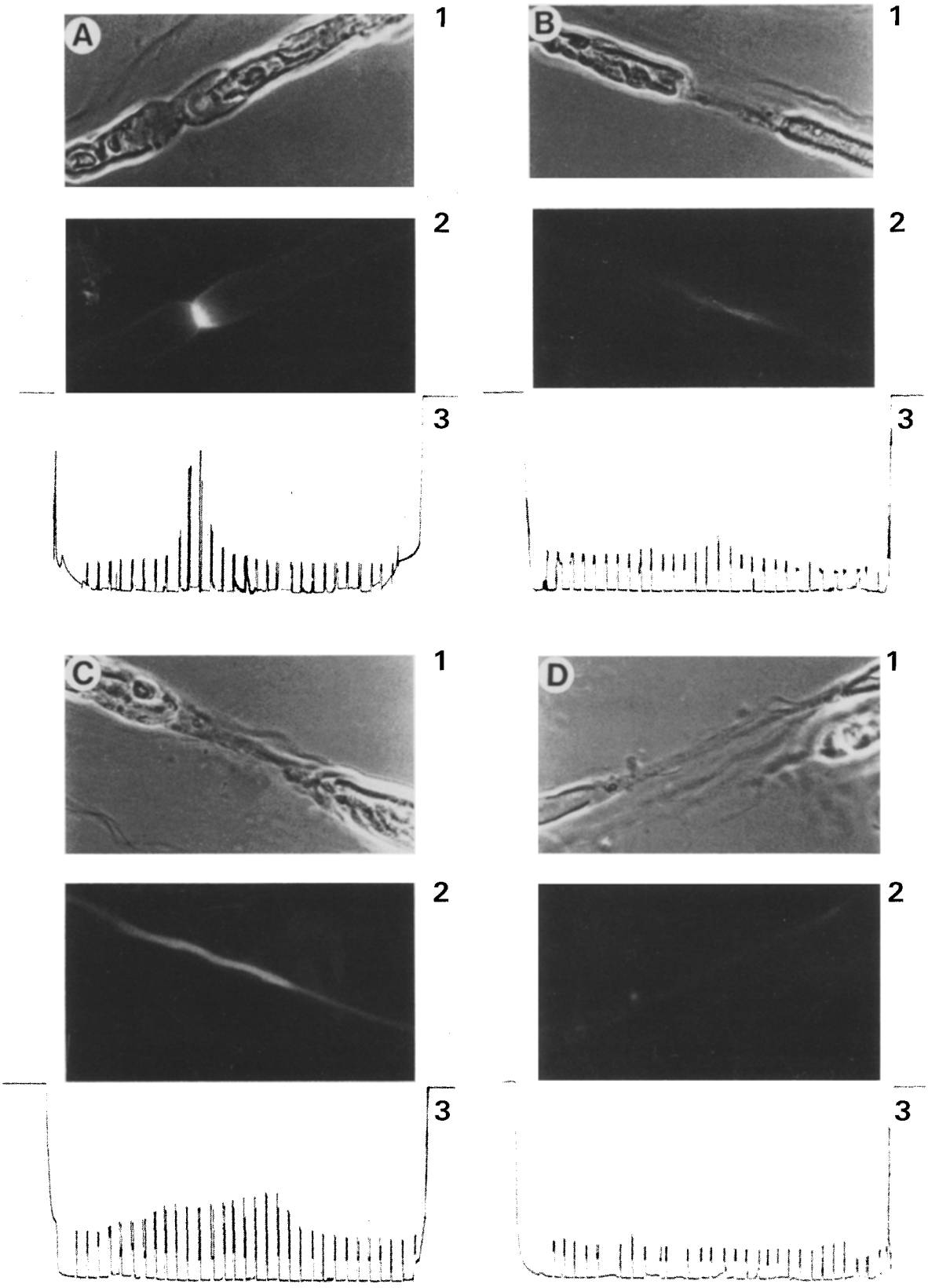

Fig. 2. Immunofluorescence labeling of single axons from rat sciatic nerve. All pictures were taken from the site of LPC or control injection. Top picture of each is a phase micrograph of a single teased axon, middle is its immunofluorescent image, bottom—light intensity measurements performed as detailed in Fig. 1. (*A*) Control myelinated axon (6 days) labeled only at the node. (*B*) Demyelinated axon from LPC treated animal (2 days) dimly labeled at the bare axolemma of internodal region. (*C*) Demyelinated axon from LPC-treated animal (6 days) brightly labeled at the bare axolemma of internodal region. (*D*) Same as *C* but labeling was performed with mAb's inactivated by pre-incubation with 10 sciatic nerves. Note: nonuniform light intensity measured along demyelinated axon at 6 days (C_3)

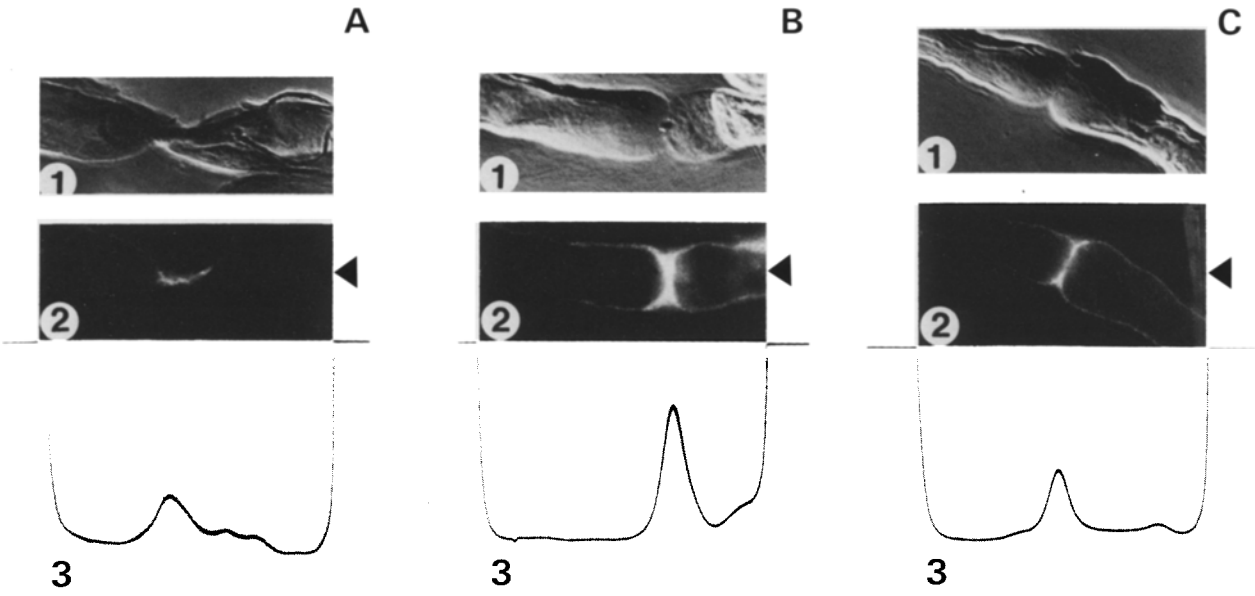

Fig. 3. Less severely affected axons. (1) Phase micrographs; (2) fluorescent image; (3) light intensity measurements along the longitudinal axis of the axon. (*A*) Paranodaly demyelinated axon (A_1) is labeled (A_2) along part of the bare axolemma. (*B*) At paranodal loosening of the myelin (B_1) labeling (B_2) is confined to nodal region only. (*C*) In unaffected axon (C_1) labeling is confined to the node of Ranvier (C_2)

sume that this labeling pattern represents an antibodies-induced channel patching. (iii) Axons labeled at the nodes of Ranvier (Fig. 3*B*, *C*) and paranodal region (Fig. 3*A*) only (very few axons). The first type of axons had long lengths of demyelinated membranes (>40 μm), while the second and third types had less.

Labeling of a large surface area of axolemma by antibodies that recognize antigenic determinants associated with sodium channel activity is consistent with continuously conducted action potentials along demyelinated axons [3]. The focal labeling of the relatively short demyelinated length of axons could represent an intermediate pattern of saltatory conduction between patches of channels at paranodal [3] or internodal [35] regions.

Elevated Number of mAb Binding Sites Measured by Radioimmunoassay

The immunofluorescence studies revealed that six days after LPC induced demyelination, an enlarged membrane surface could be labeled with sodium channel-specific mAb's. Ritchie and Rang [28] have previously shown an elevated number of ^{3}H-STX binding sites in demyelinated axons. Their finding suggests that new channels appear in demyelinated axons. In order to test if the increase of membrane surface area labeled by mAb was coupled to an elevated number of antibody binding sites, a radioimmunoassay was performed.

Our studies showed an elevation in binding site numbers for sodium channel-specific mAb's in sciatic nerves near the sites of LPC injection six days after the treatment (Fig. 4*A*, open circles) compared to contralateral nerves (Fig. 4*A*, filled circles) or control nerves (Fig. 4*B*, open circles). A smaller increase in the number of binding sites was found in control nerves (Fig. 4*B*, open circles) consistent with the finding that some demyelination occurs in control nerves (Table 2). We could not detect any significant change in the number of mAb binding sites two days after LPC injection.

In conclusion, the increased number of mAb binding sites found at six days reinforced the results showing an increase of surface area labeled by the mAb's, and it is suggested that new antigenic determinants associated with sodium channel activity appear at the region where demyelinated axons were labeled by our mAb's.

Discussion

Conduction difficulties at demyelination arise partly because the internodal axolemma is normally devoid of sodium channels [5, 30, 38] and partially because of an impedance mismatch between the normal and the demyelinated axons. At chronic de-

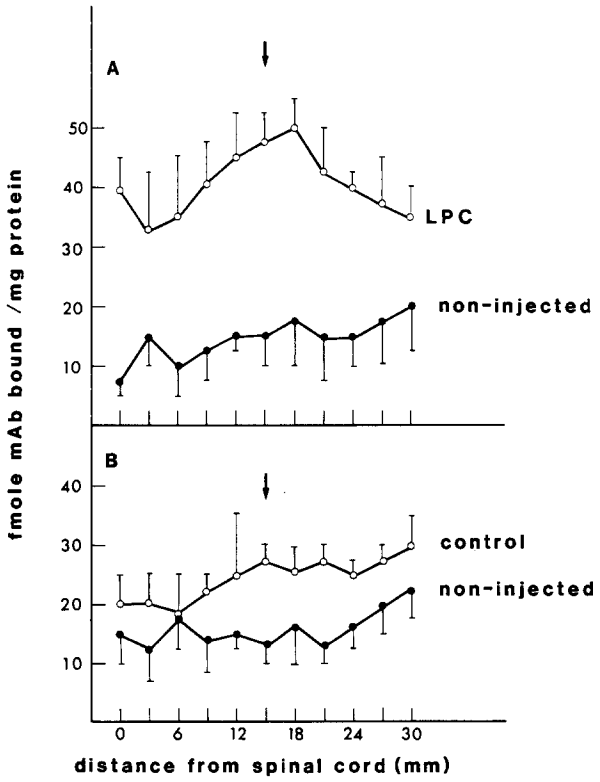

Fig. 4. Specific binding of the mAb's to sciatic nerve segments as a function of distance from the spinal cord (SP). Sciatic nerves were aligned from the spinal cord to the muscle. The specific binding was determined for each separate segment (3 mm length). (*A*) LPC-injected nerve (open circles) and the contralateral nerve (filled circles). (*B*) Control nerve (open circles) and the contralateral noninjected nerve (filled circles). Values are means of 10 nerves ($\bar{X} \pm$ SD) after background subtraction. Arrow indicates site of injection

myelination sodium channels appear at the demyelinated axons and geometrical matching can be improved [39]. In this study we examined morphologically the process of changes in sodium channel distribution developed at chronic demyelination using two monospecific antibodies capable of modifying the conductance through sodium channels.

We found that two days after exposure to LPC most axons were demyelinated. At this period, main axon population failed to conduct. Those that did conduct had slow conduction velocity. Accordingly, at this time most axons were negative when examined by immunofluorescence for the presence of sodium channels at the demyelinated region. A modified channel distribution begins to be detected by the dim labeling of demyelinated membrane found in a few axons. A previous study has shown that at acute demyelination no channels are detected at the demyelinated region [22]. On the basis of these findings we attributed the decreased amplitude of the compound action potential to conduction failure in bar axons devoid of both myelin and sodium channels. The presence of a small compound action potential carried at a relatively slower rate can, at least in part, be attributed to those axons that had already recruited sodium channels to their demyelinated axolemma. At present we cannot evaluate the precise contribution of such channel appearance to impulse recovery versus the contribution of improved geometrical "matching" that others have found [38].

Smith et al. [36] have found that six days after exposure to LPC the demyelinated axon membrane re-acquires electrical excitability. They attributed the change to recruitment of further sodium channels into the demyelinated membrane of most axons. In this study we support this view, due to the discovery of bright labeling of most of the demyelinated axons and increased numbers of mAb binding sites at six days.

Labeling pattern was nonuniform but along the entire length of the demyelinated axons. This is consistent with the continuous conduction Bostock and Sears [3] have seen in many diphtheria toxin demyelinated axons. Labeling brightness along a relatively long length of demyelinated membrane (40–60 μm) could be the immunocytochemical representation of adequate density of sodium channels required to sustain conduction through the bare axolemma. Bostock and Sears [3] have also found another group of axons demyelinated at paranodal regions only, in which widening of the nodes was compensated for by an increased density of inward nodal currents. Brismar [4] has confirmed this in voltage-clamp experiments. In this study we further support this configuration by showing the enlarged paranodal surface area labeled with our sodium channel specific mAb's.

The electrophysiological findings of Smith et al. [35] showed saltatory conduction at six days with "jumps" between sites of excitability, which they called "physiological nodes" or "Ø nodes." We did not find the exact morphological basis for Ø nodes, probably because the length of demyelinated membrane that we saw in sciatic nerve was only 40–60 μm compared to more than 200 μm which they found in spinal roots [36]. However, the recurrent foci of dense labeling (1–2 μm length) we found separated from each other by 10–15 μm along demyelinated membrane could be the immunocytochemical equivalent of their physiological or Ø nodes.

The indirect evidence for sodium channel activity at demyelinated axons [2, 3, 16, 18, 25, 35, 36] was recently proven directly by loose-patch studies [7]. Sodium current density there was smaller com-

pared to normal nodes, suggesting that demyelinated membrane has a relatively small density of channels. Further refinement of such electrical measurements and EM-immunogold labeling using our antibodies would help in characterization of ionic channel density and properties along the demyelinated region. These, together with measurements of cable properties at demyelination, may enable a complete understanding of impulse propagation at chronic demyelination [38].

The obvious question is, where are the Na channels coming from? We don't know, nor does anybody else. It has been suggested that Schwann cells [6, 33], internodal axolemma, [11, 39] and axonal transport [10] could contribute. Using the methodologies and the experimental means developed in this study, we plan to evaluate the sources and identify the signals that induce the changes in channel distribution following demyelination.

We thank Drs. M. Rasminsky, Y. Palti, Y. Schlessinger, and D. O'Dowd for fruitful discussions and helpful comments during this work as well as for revising this manuscript. We are also grateful to Miss D. Shapiro for careful typing of this manuscript and to D. Palti for photographic work. This study was supported in part by a special grant in neurobiology of the Wolf Foundation, The Israel Center for Psychobiology—Charles E. Smith Family Foundation (#054) and the Israel-US Binational Science Foundation (#84-03367).

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Received 12 December 1985; revised 6 March 1986