Alterations in the Ultrastructure of Peripheral Nodes of Ranvier Associated with Repetitive Action Potential Propagation

Charles C. Wurtz and Mark H. Ellisman

Laboratory for Neurocytology, Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, California 92093

We have studied the structure of the node of Ranvier following physiological activation and electrophysiological monitoring. Our results indicate that some structures within the nodal complex undergo microanatomic alterations concomitant with repetitive impulse propagation. The experiments were designed to determine the nature and extent of the morphologic changes and any associated changes in the compound action potential. Frog dorsal roots were examined by thin-section electron microscopy after having been either implanted, but not stimulated ("0 Hz control"), or orthodromically activated for 15 min at frequencies of 2, 5, 20, or 50 Hz. Roots were either fixed immediately and processed for electron microscopy or were allowed to recover for periods of 15, 30, or 45 min prior to fixation.

A change in nodal structure was found that correlates with the different stimulation regimens. Specifically, there was an increase in the size and number of (extracellular) paranodal intramyelinic vacuoles after stimulation at 20 or 50 Hz when compared both to the implanted nonstimulated controls and to fibers stimulated at low frequency (2 or 5 Hz). This morphologic change occurs in the vicinity of the nodal complex without similar alterations in the morphology of the internodal regions or changes in the dimensions of the nodal gap. This form of microanatomic change is reversible and is not seen if stimulation is halted and the fiber bundle allowed to recover in Ringer's prior to processing for morphologic evaluation. Changes in the compound action potential (CAP) parallel this structural alteration. The CAP is seen to slow and decrease in amplitude through the 15 min stimulation phase and then to regain its velocity and amplitude during the recovery. When TTX was applied to the preparation during stimulation the CAP was abolished and changes in the paranodal structure were not observed. Structure-function relationships indicated by these results are discussed in the context of several dynamic aspects of nodal physiology.

The node of Ranvier has been clearly established as the anatomic substrate that supports the generation of action potentials in myelinated fibers (Chiu and Ritchie, 1980a, b; Chiu et al.,

1978; Dodge and Frankenhaeuser, 1958; Huxley and Stampfli, 1949; Tasaki, 1939, 1953). With few exceptions the action potential produced focally at the amphibian node is remarkable in its similarity to that of the continuously conducting squid giant axon. Both in terms of ion selectivity and pharmacologic sensitivity, the ionic bases for both resting and action potentials are almost identical (Fritz and Brookes, 1981; Stampfli and Hille, 1976). Compact myelin is therefore generally considered to provide a passive insulation increasing transverse resistivity and decreasing membrane capacitance. This would increase the length constant of the fiber and, in the classical description of saltatory conduction, is the sole electrophysiological contribution of the myelinating cell. In this model, the paranodal region of the sheath was thought to provide the points of attachment of the Schwann cell to the axolemma (Williams and Landon, 1963), much the same role as was attributed to it by Ranvier himself in 1874.

More recent studies have resolved the architecture of the organ as a complex set of junctions between the investing Schwann cell and its axon (Berthold, 1978; Berthold and Rydmark, 1983; Ellisman et al., 1984; Peters et al., 1976; Robertson, 1959). Further advances have been made through the application of techniques such as high-voltage electron microscopy (Ellisman, 1977), freeze-fracture (Livingston et al., 1973; Wiley and Ellisman, 1980), immunocytochemical electron microscopy (Ariyasu et al., 1985; Ellisman and Levinson, 1982), microprobe ion localization (Ellisman et al., 1980; Rick et al., 1976), and laser microbeam lesioning (Mueller-Mohnsen et al., 1974). These more recent observations are forcing heuristic models of functional morphology to include a substantially increased amount of structural complexity.

Certain data suggest that, in addition to being the anatomic substrate supporting the generation of action potentials, the node may be involved in determining various physiological parameters of these action potentials. Moreover, these parameters appear to change through time with repetitive activity. Some of these "aftereffects" have time courses of 20 min or more, suggesting that some structural changes may be occurring (Goldstein, 1978; Raymond and Lettvin, 1978; Swadlow and Waxman, 1978).

Several studies have examined structural changes in this organ following electrical stimulation. The first (Zagoren and Arczzo, 1982) reports that a short period of 200–500 Hz stimulation alters the staining pattern of Hatchett's brown reaction product in myelinated rat sciatic fibers. The quiescent control fibers predominantly stain at the paranode, while the stimulated fibers predominantly stain the node proper. Allowing a 15 min recovery prior to fixation produces staining in the unstimulated pattern. Padron and Mateu (1982), using X-ray diffraction, next detailed the structural effects on toad myelin produced by high-frequency activity. They noted that following prolonged 200 Hz activity myelinated fibers were biased toward osmotically

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Correspondence should be addressed to Dr. Mark H. Ellisman, Laboratory for Neurocytology, Department of Neurosciences, M-008, School of Medicine, University of California, San Diego, La Jolla, CA 92093.

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Figure 1. Diagram of the electrophysiologic preparation used to activate dorsal roots either directly, or via the sciatic nerve, while maintaining continuous superfusion. Following stimulation (or recovery), the flow of Ringer's solution is replaced with cacodylate-buffered fixative. See Materials and Methods for details.

induced swelling of the intraperiod lines. Finally, Moran and Mateu (1983) examined the sensitivity of mammalian nodes to 4-aminopyridine, both before and after the supramaximal stimulation regimen, to look for physiologic changes that might be reflective of loosened paranodal myelin. In these experiments, compound action potentials (CAPs) from rat sciatic nerves show substantial sensitivity to this potassium channel blocker following stimulation. This suggests that high-frequency activity disrupts paranodal myelin, somehow exposing potassium channels that then become instrumental in regulating action potential characteristics.

The preceding evidence indicates that some component of the paranodal apparatus is influenced by electrical activity, which, in turn, functions to modulate the potassium currents elicited during the generation of subsequent action potentials. This paper examines the ultrastructure of the node-paranode region to elucidate the microanatomical changes that this organ undergoes during and after repetitive activation and the electrophysiologic effects that temporally occur simultaneously with them.

Materials and Methods

Rationale

Two stimulation methods, direct and sciatic (see Physiological stimulation, below), were designed to provide a segment of dorsal root that had been subjected to a controlled stimulation regimen and ionic environment. Both preparations allowed the physiologic response to the stimulation to be recorded and, following the stimulation (or recovery), allowed the nerve bundle to be rapidly fixed *in situ*.

Surgical procedure

Frogs, 10–12 cm *Rana catesbeiana* (Central Valley Biological, Clovis, CA), were maintained in stainless-steel tanks and fed goldfish twice per week until used in an experiment. Animals were stunned by a blow to the head, rapidly decapitated, and pithed to the level of C₁. A laminectomy was performed to expose the spinal cord and dorsal roots to

the level of the filum terminali. In those experiments utilizing sciatic stimulation, the sciatic nerve was exposed on one side. The nerve was freed from its connective tissue and blood vessels but was not transected until after it was attached to the stimulating electrode. After dissection, the frog was pinned to a Silgard-coated dish and placed in the electrophysiology apparatus (modified from Zachar, 1967). The root to be stimulated was supported by a Teflon spatula shaped like the inverted nib of a quill pen. This was connected to a peristaltic pump providing a constant flow of tempered ($t = 17^{\circ}\text{C}$) 0.12 M oxygenated frog Ringer's (pH 7.3). After superfusing the suspended segment of the spinal root, the Ringer's was directed into the vertebral column, superfusing the contralateral control root and spinal cord. Isopotentiality between the main pool and the spatula was maintained by connecting them with a chlorided silver wire. (See Fig. 1 for schematic diagram of the physiology preparation.) Following the stimulation regimen, or recovery phase, the superfusion media was exchanged for fixative (2% paraformaldehyde, 4% glutaraldehyde in 0.12 M cacodylate buffer at pH 7.3 at a temperature of $t = 17^{\circ}\text{C}$). Roots were thus fixed *in situ* for 10 min and were then excised and placed in fresh cold (4°C) 2% glutaraldehyde buffered with 0.12 M cacodylate for 12 hr.

Physiological stimulation

Direct dorsal root stimulation

In this protocol the root to be stimulated was picked up near its point of entry into the cord by a fine chlorided silver wire (recording) hook electrode, and near the intervertebral foramen by a fine stainless-steel bipolar (stimulating) hook electrode. The indifferent electrode was attached to a forelimb. The central segment of the root (about 1.0 cm) was supported by the Teflon spatula. The stimulus level was determined by increasing the voltage while holding the pulse duration constant at 0.025 msec until further increase did not change the amplitude of the CAP. The experimental stimulus was then set at twice this value, generally between 10 and 12 V. In three 50 Hz experiments, 12.5 μM TTX was introduced into the superfusion medium to abolish the CAP as a control for stimulation artifact.

In those experiments where fixation was to immediately follow stimulation, the flow of fixative was introduced and the stimulator was kept running until the CAP decremented. In the recovery series, the stimulator was turned off at the end of the 15 min stimulation phase and the roots left in the Ringer's bath. At the end of the recovery, 1 or 2 test pulses were delivered to the nerve to record the recovered CAP prior to the introduction of fixative.

Activation of dorsal roots by sciatic stimulation

To further preclude the possibility of stimulation artifact, and also to enhance the resolution of CAP components, a series of experiments was performed in which dorsal roots were activated by stimulating the sciatic nerve approximately 10 cm distal to the segment of root to be used for ultrastructural analysis. As stated above, the bipolar stimulating electrode ("miniature bipolar stimulating electrode," Harvard Bioscience) was attached to the nerve, and 1 or 2 test pulses were delivered. Preparations in which the test pulses failed to elicit both muscle twitch and dorsal root CAP were deemed to be surgically damaged and were not used for analysis. Following the test pulses, the sciatic nerve was transected distal to the stimulating electrode to prevent movement of the animal during stimulation. The superfusion apparatus and recording electrodes were placed as in the direct method. Stimulation was provided by a Grass S88 multifunction stimulator. The electrical responses were recorded with a Grass P-9 AC preamplifier and displayed on Tektronix 565 and 564 oscilloscopes. Permanent records of the compound action potentials were taken by photographing traces at 5 min intervals from the 564 storage scope. Parameters for sciatic stimulation were determined as in the direct dorsal root stimulation protocol. As before, primary fixation was achieved by replacing the Ringer's media with fixative.

Analysis of compound action potentials

The analysis of electrophysiological records was restricted to the conduction velocity and overall amplitude of the CAP. In the case of sciatic nerve stimulated preparations, these same observations were made on the several separate CAP components. Changes in conduction velocity for the direct dorsal root stimulated preparations were ascertained by measuring the latency between the stimulus artifact and the crossover

Figure 2. *A*, Large, nonstimulated Phillips type II node of Ranvier from a frog dorsal root. Note the general integrity of the compact myelin within the paranodal apparatus and the large infolded stacks of small osmiophilic cytoplasmic loops not in contact with the axolemma. Vacuoles like the one visible within a single paranodal region of this node are occasionally found in unstimulated fibers of very large diameter. $\times 18,000$. *B*, Nodal and paranodal regions of a small Phillips type I node from a nonstimulated control root. Sequential apposition of paranodal loops to the axolemma are evident in this micrograph. $\times 41,000$. *C*, Nodal and paranodal regions of a medium-sized Phillips type II node from a nonstimulated control root. Dotted outline of one of the nodal gaps illustrates the perimeter criteria used in assessing changes in nodal gap geometry (see also Fig. 7). $\times 10,000$.

Figure 3. *A*, Focal nature of the stimulation-induced vacuolization is evident in this low-magnification electron micrograph. This fiber is from a preparation activated by direct dorsal root stimulation at 50 Hz for 15 min. Note that the adjacent fiber displays an intact Schmidt-Lanterman cleft (arrows) and fully intact internodal compact myelin. $\times 5500$. *B*, Node from a large sensory fiber stimulated at 50 Hz for 15 min. Large intramyelinic vacuoles are present in both the proximal (right) and distal (left) paranodes. Note that despite the severity of the paranodal disruption, the nodal gap remains essentially unaffected. $\times 12,100$.

points of the initial negativity and final positivity. For the sciatic stimulated preparations the latencies between the stimulus artifact and the various peaks of the CAP were measured. Since the exact length of the stimulated nerve was not determined, only relative values were obtained. The results are thus restricted to comparisons among the CAPs recorded during the various stages of the stimulation regimens. No attempt was made to compare CAP parameters across animals.

Specimen preparation

After excision, both the stimulated root and the contralateral nonstimulated control root were placed in 4°C buffered 2% glutaraldehyde for 12 hr. The tissue was then washed in buffer and postfixed in buffered 1% osmium tetroxide for 30 min, stained *en bloc* in 0.5% aqueous uranyl acetate for 2 hr, dehydrated in a series of ethanols, and finally infiltrated and embedded in Epon-Araldite. Blocks were trimmed to optimize longitudinal axon orientation and sectioned on an A. O. Reichert Ultracut or Ultracut E. Ten sections of silver interference (60–70 nm) were taken every 15 μ m through the block and grid-stained with uranyl acetate and lead prior to examining them with a JEOL 100 CX EM operated at 80 kV.

Ultrastructural analysis

The embedded dorsal roots were sectioned until either micrographs of 30 nodes from each experimental condition were obtained, or until the

sectioning had progressed completely through the nerve. For each node a single micrograph from a longitudinal section near the midline was selected for quantification. The diameter of the fiber and the myelin thickness were measured at the paranode–internode interface. The degree of intramyelinic vacuolization was then determined by tracing the perimeter of each of the resolved “arms” of the paranodal apparatus on a computer-interfaced Summagraphics digitizing tablet. Each vacuole was then traced in a similar manner, and the ratio of vacuole area to the total area of the paranodal apparatus was computed (creating the parameter “percent vacuolization”).

The geometry of the nodal gap was determined by tracing the perimeter of the gap as delimited by the axolemma, the Schwann cell cytoplasmic collars, and the basal lamina (see outlined nodal gap in Fig. 2C) on the Summagraphics tablet. The computer then determined the area and perimeter. These 2 parameters were then used to compare the shape of the nodal gap between stimulated, nonstimulated, and stimulated-recovered fiber bundles. (The various possible nodal gap geometries are illustrated in Fig. 7.)

Statistical analyses

Paranodal vacuolization

The degree of vacuolization was established by subtracting the mean percent vacuolization of the contralateral control roots from the per-

Figure 4. Gallery of paranodal regions from fibers stimulated as in Figure 3*A* and *B* above. *A*, Low magnification of a relatively large fiber exhibiting 3 vacuolized paranodes. The adjacent paranodes in the upper portion of this micrograph are presented at higher magnifications in panels *C–E*. *B*, Another example of paranodal vacuolization of an intermediate-sized fiber. Note that the paranodal axoglial junctions remain attached despite the disruption. *E*, Region of the paranode at higher magnification showing a series of myelin terminal loops in which the internal membrane surfaces are still closely apposed, forming the myelin major dense lines. The extracellular surfaces, however, are separated at what were the myelin minor dense lines (arrows). *F*, High-magnification micrograph demonstrating that the major dense line of the compact myelin remains intact as the vacuole forms. The separations occur instead in the space between the myelin lamellae resulting in expansion of the extracellular space within the paranodal apparatus. Magnification: *A*, $\times 5500$; *B*, $\times 10,500$; *C* and *D*, $\times 22,500$; *E*, $\times 48,000$; *F*, $\times 250,000$.

Figure 5. Bar graphs showing the percent vacuolization occurring after direct dorsal root stimulation under a variety of conditions (see labels on abscissa). All these results are statistically collapsed across fiber diameter. Error bars indicate $\pm$ SEM.

centage vacuolization of the stimulated roots. This figure, S_{vac} was compared with the value C_{vac} which was obtained from the implanted nonstimulated root and its contralateral control. *Post hoc* statistical comparisons were made between the groups using the Newman-Keuls test.

Geometry of the nodal gap

A Student's *t* test for 2 means was performed on the parameter generated by the ratio of gap perimeter to gap area.

Results

Morphological changes in node structure following activity

A focal disruption of the paranodal apparatus that varies in magnitude according to the frequency of activation is found following extended electrical stimulation. Morphologically, this disruption appears in the form of extracellular vacuoles within the paranodal apparatus and involves a splitting of the minor dense line of the compact myelin. (Compare Fig. 2 with Figs. 3, 4.) This appears to occur without disruption of either the axoglial junction between the paranodal loops and the axolemma or the *zonula adhaerens* that joins together adjacent helical wraps of the lateral cytoplasmic loop. Sections containing desmosomal junctions linking sequential paranodal loops revealed no disruption of the junction itself, although often displaying vacuoles in adjacent areas. This indicates that these structures

Figure 6. Bar graphs showing the degree of sciatic stimulation-induced paranodal vacuolization during the recovery from activation at 50 Hz for 15 min. As in Figure 5 these results were collapsed across fiber diameter. Error bars indicate $\pm$ SEM.

are retained during stimulation-associated vacuolization. It is noteworthy that the splitting of the minor dense line generally terminates at or before the paranodal/internodal interface, even in the most vigorously stimulated fibers. Thus, stimulus-induced vacuolization appears to be restricted to the paranodal apparatus (Fig. 3A). No difference in the effect is evident between the proximal and distal paranodal bulbs.

Quantitative evaluation of the extent of changes in node morphology relative to stimulation frequency

When stimulation was applied directly to the dorsal roots, the magnitude of the paranodal vacuolization effect was substantial. The 20 Hz fibers exhibited a 6.3-fold greater vacuole ratio than the 0 Hz control ($p < 0.05$) group and an approximately 3.1-fold greater vacuole ratio than either of the low-frequency groups (2 or 5 Hz; $p < 0.05$). The effect at 50 Hz was even more dramatic, with activated nodes averaging over 9.5-fold greater vacuole ratio than nonstimulated controls ($p < 0.03$), a 5.3-fold greater vacuole ratio than the low-frequency stimulated group ($p < 0.05$) and a 1.5-fold greater vacuole ratio than the 20 Hz stimulated group ($p < 0.05$). Although a slight increase in vacuolization is evident, the difference between the low-frequency group and the 0 Hz group is not statistically significant. In no cases did the contralateral unimplanted controls differ significantly from the 0 Hz implantation controls.

In the sciatic stimulated preparations, nodes activated at 50 Hz display a 4.8-fold increase in vacuole ratio compared to their unstimulated controls. This difference proved significant at the $p < 0.03$ level.

In both the direct and sciatic stimulated protocols, addition of 12.5 μ M TTX to the superfusate blocked the vacuolization response. Neither simple visual inspection of the micrographs nor computerized planimetry reveals any detectable morphologic differences between the 50 Hz-stimulated/TTX-blocked fibers and the nonstimulated controls. The results from each stimulation protocol are displayed graphically in Figures 5 and 6.

While this study did not directly assess the morphologic com-

Figure 7. Diagrams illustrating possible area:perimeter (dotted outline) relationships at the nodal gap. *A*, The most common appearance of the gap. *B*, The area is minimized while maintaining approximately the same perimeter as in *A*. *C*, Perimeter and area are both minimized. *D*, The area is maximized while holding the perimeter to the same approximate length as in *A* and *B*. For simplicity, the diagrams illustrate only a Phillips type I node. The principle, however, is the same for all fibers (see text).

ponents of the nodal gap (such as the distribution and microanatomy of the microvilli), general measurements of nodal gap parameters were taken. If substantive deformation of the nodal gap were occurring during impulse propagation, a logical and simple measure would be the area:perimeter ratio. Figure 7 depicts several possible nodal gap geometries, all of which are found to some extent in dorsal root nodes of Ranvier. Analysis of the nodal gap area:perimeter ratio, however, indicates that this distribution of structures does not change significantly following stimulation (Fig. 8). Finally, the integrity of the compact myelin, Schmidt-Lanterman clefts, and periaxonal spaces within the internodal region shows no morphologic differences from controls (Fig. 3A).

Influence of fiber diameter on the morphologic response

Stimulation both at 20 and 50 Hz appears to effect the paranodal morphology of larger fibers to a significantly greater extent than medium- and small-diameter fibers; in particular, small fibers with Phillips type I morphology (Phillips et al., 1972), such as the control node in Figure 2B, are unaffected. A scatter plot of the percent vacuolization versus fiber diameter, as in Figure 9,

Figure 8. Scatter plot indicating the lack of significant change in the geometry of the nodal gap following stimulation at 50 Hz for 15 min.

demonstrates that larger fibers contribute disproportionately to the effect. While fiber diameter may bias larger nodes toward activity-induced disruption, it apparently does not predispose large fibers to fixation artifact. This is suggested by the consistently low level of paranodal vacuolization demonstrated by the controls. In the very largest fibers ($D > 12 \mu\text{m}$), however, 1 or 2 small vacuoles are normally present regardless of condition.

Changes in the physiological response of fibers over the stimulation interval

The roots stimulated at 50 Hz (and, to a lesser degree, 20 Hz) show a reduction in the conduction velocity of the CAP, which is accompanied by a broadening of both the positive and negative phases and a depression in the overall (peak-to-peak) amplitude (see Figs. 10 and 11 for representative traces of both direct and sciatic stimulated roots).

Since the exact length of the nerve could not be adequately measured, only relative changes in conduction velocities were determined. This was accomplished by measuring the distance on the oscillographic trace from the stimulus artifact to the initial positive peak and from the artifact to the crossover point. These distances (latencies) were compared at the beginning and end of stimulation and following the recovery period. In general, the 50 Hz results (regardless of stimulation locus) show that the CAP undergoes an overall reduction in conduction velocity of $30 \pm 10\%$ at the end of the 15 min stimulation regimen. In the directly elicited CAP, this reduction is accompanied by a depression in the overall amplitude averaging $40 \pm 15\%$.

The changes in amplitude vary as a function of conduction velocity in the sciatic stimulated roots. In these traces, the peaks representing the faster conducting fibers exhibit a $70 \pm 15\%$ reduction in amplitude, while the peaks representing the slowest conducting (myelinated) fibers evidence a $40 \pm 10\%$ reduction.

More detailed observations on the nature of changes in components of the CAP were difficult to obtain within the constraints of the recording system used. However, further changes do include an increase in the rise time of the initial CAP component averaging $10 \pm 6\%$, and alterations in the final falling phase which proved too variable to quantify. While it is true that monophasically recorded CAPs would yield a waveform much

Figure 9. Scatter plot showing the interaction between fiber diameter and the extent of stimulation-induced vacuolization. Note the overlap in the percent vacuolization in the smaller diameter fibers.

more amenable to this kind of analysis, we found the use of a crush electrode problematic for good preservation of morphology. For this reason, all experiments from which these observations were obtained were conducted using biphasic recording.

Morphology and physiology of stimulated nerves following a recovery interval

Morphology

When stimulation is halted prior to fixation and the fibers allowed to recover, the vacuolized appearance of the paranodal apparatus that occurs with high-frequency stimulation is not seen. Instead, the paranode appears relatively nonvacuolized, resembling the nonstimulated fibers found both in the intact contralateral controls and in the implanted but not stimulated roots (see Fig. 12).

This return to "baseline" paranodal morphology occurs over the course of 30 min following the cessation of electrical stimulation. The recovery proceeds similarly regardless of whether the roots have been activated directly or via sciatic nerve stimulation. Although direct stimulation of the dorsal roots consis-

tently produced a more robust vacuolization response, the percent vacuolization measured at each time point shows that the recovery is independent of the locus of stimulation. The bar graphs in Figures 5 and 6 show that the morphology of the paranodal region still differs from that of the controls after 15 min of recovery ($p < 0.05$ in both preparations). After 30 min, however, no significant difference is apparent.

Physiology

Using the criteria of return of relative CAP conduction velocity and of CAP amplitude, it is clear that the morphologic recovery precedes the physiologic recovery by several minutes. Regardless of stimulation protocol, morphologic recovery from 15 min of 50 Hz stimulation was essentially complete by 30 min, while full return of prestimulation conduction velocity was not obtained until 45 min. Complete recovery to prestimulation amplitude was not seen. This is apparently due to a potentiation of the CAP amplitude (and to a lesser extent the conduction velocity), such that the initial values are overshoot by several percent (see panels D in Figs. 10 and 11). As we did not perform experiments utilizing recovery times greater than 45 min, we

Figure 10. Compound action potential recordings from a directly activated frog dorsal root. The fiber bundle is being stimulated at 50 Hz in frames A–C, which represent the time points 0, 10, and 15 min (end of stimulation phase). Arrows in B–D indicate peak separation. Note that the CAP sequentially decrements in amplitude and slows in conduction velocity during the stimulation phase, but significantly recovers over a period of 30 min. The final frame shows the waveform of the CAP following recovery in 0.12 M frog Ringer's. Arrowheads indicate the stimulation artifact. Scale, 5 mV/1 msec per division.

Figure 11. Compound action potential recordings from a sciatic stimulated dorsal root. The components of the CAP are easily seen, as are the depressed amplitude and reduced conduction velocity. As in Figure 10, the stimulation parameters are 50 Hz for 15 min; frames A–C represent time points 0, 10, and 15 min. The final frame is the trace recorded after a 45 min recovery. Note that the recovery time point displays a CAP that has fully recovered its conduction velocity. In addition, the amplitude of the fastest CAP component appears to be potentiated such that it has actually overshoot the initial conditions. As in Figure 10, arrowheads indicate the stimulation artifact. Scale, 5 mV/1 msec per division.

do not know the length of the full recovery time course that amphibian peripheral CAPs would exhibit under our experimental conditions. Clearly, however, neither the fiber dropout nor the slowed conduction velocity present at the end of the stimulation phase is reflected in the CAP recorded after 45 min of recovery.

Discussion

Sources of artifact

Evidence that “normal” physiological action potential generation is actually inducing the vacuolization, rather than its being

Figure 12. Frog dorsal root node of Ranvier following 45 min of recovery from 15 min of 50 Hz activity. This particular node is from the experimental preparation whose electrophysiological recordings are shown in Figure 11 and received sciatic stimulation. $\times 18,000$.

an artifact of the 10–11 V pulses produced by the stimulator, is provided both in those experiments in which the roots are superfused with TTX during a 15 min 50 Hz train and in those in which the dorsal roots are activated via the sciatic nerve, on average, 7–8 cm distant from the area of nerve to be morphologically evaluated. The TTX protocol abolishes the sodium phase of the action potential and prevents saltatory conduction. Therefore, the only depolarizing current is the electrotonic wave originating at the stimulating electrodes. This creates a much lower current density than normally obtained at the nodal membrane, and thus potassium currents should be significantly reduced along with the sodium currents. Sciatic stimulation simply removes the stimulus sufficiently from the assayed region, such that artifactual longitudinal current is negligible.

A heuristic model for the morphologic effects

Two assumptions are necessary to create a model that explains the morphologic alterations associated with repetitive nodal activity: (1) Potassium is assumed to carry the repolarizing current at the node, and (2) the paranodal axolemma is the site of potassium efflux during the repolarization phase of the action potential. Given these assumptions, several models can be constructed to explain the morphologic and physiologic data.

For example, we could consider the vacuolization phenomenon as occurring in the following manner: As suggested by Brismar (1983) and implied by the data of Binah and Palti (1981), some of the electrophysiologically "cryptic" potassium current may flow directly from the axon into the paranodal loops in a manner similar to the direct axoglial electrical coupling reported in squid giant axon by Villegas (1972). If the remainder passes through potassium channels that are not directly coupled to the Schwann cell, it will end up in the periaxonal space (Dubois and Bergman, 1975).

Such differential shunting could be due to multiple populations of kinetically and pharmacologically distinct potassium conductances. Evidence for the existence of at least 2 such entities at frog nodes has been obtained by Ilyin et al. (1980) and Dubois (1981). Possibly these represent coupled versus non-coupled channels.

In this model, the axoglial electrical coupling of the potassium current is to a certain extent "leaky," especially in large type II fibers. The ratio of directly shunted potassium to indirectly uptaken potassium would probably be proportional to the ratio of cytoplasmic loop in contact with the axolemma; that is, it would vary with fiber diameter. Additionally, mechanisms for paranodal Schwann cell uptake and sequestration of extracellular potassium would saturate at some given rate of ion transport. At low operating frequencies, the 2 systems would be sufficient to prevent potassium from accumulating as it is extruded by the axon. When the frequency is increased, however, the rate of potassium efflux would exceed the buffering capability of the paranodal apparatus. Potassium accumulation would thereby produce an increased degree of hypertonicity. This hypertonic condition of the periaxonal space could then result in an influx of water from the interstitium and cause a localized microedema that could hydraulically split the compact myelin at the minor dense line.

Another factor that might disrupt the morphology of the activated paranode (and possibly further explain its dependence on fiber diameter) is the heat evolved by the generation of action potentials. Howarth and coworkers (1968), who measured the heat produced by the passage of single action potentials along unmyelinated fibers of the rabbit vagus nerve, found that a substantial increase in temperature (approximately 200 $\mu^\circ\text{C}$ /spike) occurs concomitant with activity. More pronounced heating might occur in myelinated fibers at nodes of Ranvier, where action current densities are thought to be over an order of magnitude greater than occur along unmyelinated C fibers and heat

dissipation is hampered by upwards of 100 layers of compact myelin. Since small fibers possess fewer myelin lamellae, produce less current per action potential, and have a greater surface-to-volume ratio for heat dissipation, smaller fibers might operate at a lower temperature than large fibers. [A number of studies, among them Paintal (1966) and Smith and Schaaf (1981), have described the various effects of fiber diameter on the physiological properties of myelinated axons.]

These factors could combine to produce the frequency threshold in the vacuolization effect, described in Figure 5. This model may also explain the disappearance of vacuoles following recovery: During a quiescent phase, potassium uptake reduces the osmolarity of the periaxonal space, while convection and conduction cooling return the node to its resting temperature. Further, the model suggests why Rosenbluth (1983) found no discernible changes in the membrane morphology of activated versus anesthetized nodes. First, small type I nodes appear to be virtually unaffected. Second, we detect little or no disruption of the axoglial junctions even in the most vacuolized cases. These 2 observations, coupled with the fact that freeze-fracturing preferentially exposes nodes of small-diameter fibers, suggest that studies of membrane architecture performed by this method would be less likely to detect substantial effects.

Changes in the physiological response

Several of the possible factors implicated in the morphologic effects could also produce the alterations seen in the traces of the CAPs. It is well established that potassium accumulation and increases in the potassium conductance can raise the excitability threshold for the initiation of nodal action potentials (Huxley and Stampfli, 1951; Stampfli and Hille, 1976). This has the effect of reducing the safety factor and also depressing conduction velocity. The reduced amplitude of the CAP, on the other hand, is likely to be the result of intermittent firing of individual axons within the fiber bundle. Since the recorded CAP is a sum of the ionic events occurring at nodes within the dorsal roots, phase-locked intermittent activity by individual myelinated axons would produce an electrophysiologic recording similar in appearance to one resulting from a reduction in the amplitude of the action potential of each of the constituent nodes. These phenomena have been well characterized in peripheral nerve bundles by Raymond and Lettvin (1978).

An indication that rapidly occurring paranodal disruption can be a factor in electrophysiologic impairment of this nature is found in the work of Saida and coworkers (1984). These investigators noted that antigalactocerebrosidase-mediated disruption of the paranodal apparatus was paralleled by reduced efficacy of nerve conduction. Their electron micrographs reveal a paranodal morphology that is remarkably similar to the vacuolized paranodal myelin described in the present study. A further suggestion that pathologically altered paranodal morphology is associated with electrophysiological dysfunction was recently reported for spontaneously diabetic rats by Sima and Brismar (1985).

Consideration of experimental methodology

It is not determinable within this experimental design whether these morphologic changes are actually occurring in the living fiber, or if the stimulation effects are simply predisposing the fiber to fixation artifact. Padron and Mateu's (1982) study suggests the latter may be the case, in that they saw no changes in the X-ray diffraction pattern of amphibian myelin during stimulation but only after the bathing media had been replaced with distilled water. Conversely, the changes in the accessibility of paranodal potassium channels to tetraethylammonium (TEA) and 4-aminopyridine in the rat occur during stimulation, while the nerve bundle is maintained in normal Ringer's (Moran and Mateu, 1983). Currently, we are examining stimulated fibers

fixed by rapid freezing/freeze substitution and have obtained evidence that vacuolization is seen regardless of the method of tissue preparation (Wurtz and Ellisman, 1985). This strongly suggests that this structural alteration occurs *in vivo*.

References

- Ariyasu, R. G., M. H. Ellisman, J. A. Nichol, and T. J. Deerinck (1985) Immunocytochemical localization of sodium-potassium adenosine triphosphatase in the rat central nervous system. *J. Neurosci.* 5: 2581–2596.
- Berthold, C.-H. (1978) Morphology of normal peripheral axons. In *Physiology and Pathobiology of Axons*, S. G. Waxman, ed., Raven, New York.
- Berthold, C.-H., and M. Rydmark (1983) Anatomy of the paranode–node–paranode region in the cat. *Experientia* 39: 964–976.
- Binah, O., and Y. Palti (1981) Potassium channels in the nodal membrane of rat myelinated fibers. *Nature* 290: 598–599.
- Brismar, T. (1983) Nodal function of pathological nerve fibers. *Experientia* 39: 946–953.
- Chiu, S. Y., and J. M. Ritchie (1980a) Potassium channels in nodal and internodal axonal membrane of mammalian myelinated fibers. *Nature* 284: 170–171.
- Chiu, S. Y., and J. M. Ritchie (1980b) Potassium channels in the paranodal region of acutely demyelinated voltage-clamped mammalian myelinated nerve. *J. Physiol. (Lond.)* 305: 61P.
- Chiu, S. Y., and J. M. Ritchie (1981) Evidence for the presence of potassium channels in the paranodal region of acutely demyelinated mammalian single fibres. *J. Physiol. (Lond.)* 313: 415–437.
- Chiu, S. Y., J. M. Ritchie, R. B. Rogart, and D. Stagg (1978) A quantitative description of membrane currents in rabbit myelinated nerve. *J. Physiol. (Lond.)* 292: 149–166.
- Dodge, F. P., and B. Frankenhaeuser (1958) Membrane currents in isolated frog nerve fibers under voltage clamp conditions. *J. Physiol. (Lond.)* 143: 76–90.
- Dubois, J. M. (1981) Evidence for the existence of three types of potassium channels in the frog Ranvier node membrane. *J. Physiol. (Lond.)* 318: 297–316.
- Dubois, J. M., and C. Bergman (1975) Potassium accumulation in the perinodal space of frog myelinated axons. *Pflügers Arch.* 358: 111–124.
- Ellisman, M. H. (1977) High voltage electron microscopy of cortical specializations associated with membranes at nodes of Ranvier. *J. Cell Biol.* 75: 108a.
- Ellisman, M. H., and S. R. Levinson (1982) Immunocytochemical localization of sodium channel distributions in the excitable membranes of *Electrophorus electricus*. *Proc. Natl. Acad. Sci. USA* 79: 6706–6711.
- Ellisman, M. H., P. L. Friedman, and W. J. Hamilton (1980) The localization of sodium and calcium to Schwann cell paranodal loops at nodes of Ranvier and of calcium to compact myelin. *J. Neurocytol.* 9: 185–205.
- Ellisman, M. H., C. A. Wiley, J. D. Lindsey, and C. C. Wurtz (1984) Structure and function of the cytoskeleton and endomembrane systems at the node of Ranvier. In *The Node of Ranvier*, J. C. Zagoren and S. Federoff, eds., Academic, New York.
- Fritz, L. C., and J. P. Brookes (1981) Clustering of ion channels at the node of the Ranvier. *Nature* 291: 190.
- Goldstein, S. S. (1978) Models of conduction in non-uniform axons. In *Physiology and Pathobiology of Axons*, S. G. Waxman, ed., Raven, New York.
- Howarth, J. V., R. D. Keynes, and J. M. Ritchie (1968) The origin of the initial heat associated with a single impulse in mammalian non-myelinated nerve fibres. *J. Physiol. (Lond.)* 194: 745–793.
- Huxley, A. F., and R. Stampfli (1949) Evidence for saltatory conduction in peripheral myelinated nerve fibers. *J. Physiol. (Lond.)* 108: 315–339.
- Huxley, A. F., and R. Stampfli (1951) Effect of potassium and sodium on resting and action potentials of single myelinated fibers. *J. Physiol. (Lond.)* 112: 496–508.
- Ilyin, V. I., I. E. Katina, A. V. Lonskii, V. S. Makovski, and E. V. Polishchuk (1980) The Cole-Moore effect in nodal membrane of the frog *Rana ridibunda*: Evidence for fast and slow potassium channels. *J. Membr. Biol.* 57: 179–193.
- Livingston, R. B., K. Pfenninger, H. Moor, and K. Akert (1973) Specialized paranodal and interparanodal glial-axonal junctions in the peripheral and central nervous system: A freeze-etching study. *Brain Res.* 58: 1–24.
- Moran, O. and L. Mateu (1983) Loosening of paranodal myelin by repetitive propagation of action potentials. *Nature* 304: 344–345.
- Mueller-Mohnsien, H., A. Tippe, F. Hillenkamp, and E. Unsöld (1974) Is the rise of the action potential of the Ranvier node controlled by a paranodal organ? *Naturwissenschaften* 61: 369–370.
- Padron, R., and L. Mateu (1982) Repetitive propagation of action potentials destabilizes the structure of the myelin sheath. *Biophys. J.* 39: 183–188.
- Paintal, A. S. (1966) The influence of diameter of medullated nerve fibres of cats on the rising and falling phases of the spike and its recovery. *J. Physiol. (Lond.)* 184: 791–811.
- Peters, A., S. L. Palay, and H. de F. Webster (1976) *The Fine Structure of the Nervous System*, Saunders, Philadelphia, PA.
- Phillips, D. D., R. G. Hibbs, J. P. Ellison, and H. Shapiro (1972) An electron microscopic study of central and peripheral nodes of Ranvier. *J. Anat.* 111: 229–238.
- Ranvier, L.-A. (1874) De quelques faits relatifs à l'histologie et à la physiologie des muscles striés. *Arch. Physiol. Norm. Pathol.* 2 Sér. 1: 5–15.
- Raymond, S. A., and J. Y. Lettvin (1978) Aftereffects of activity in peripheral axons as a clue to nervous coding. In *Physiology and Pathobiology of Axons*, S. G. Waxman, ed., Raven, New York.
- Rick, R., A. Doerge, and A. Tippe (1976) Elemental distribution of Na, P, Cl and K in different structures of myelinated nerve of *Rana esculenta*. *Experientia* 32: 1018–1019.
- Robertson, J. D. (1959) Preliminary observations on the ultrastructure of nodes of Ranvier. *Z. Zellforsch.* 50: 553–560.
- Rosenbluth, J. (1983) Intramembranous particle distribution in nerve fiber membranes. *Experientia* 39: 953–963.
- Saida, K., T. Saida, H. Kayama, and H. Nishitani (1984) Rapid alterations of the axon membrane in antibody-mediated demyelination. *Ann. Neurol.* 15: 581–589.
- Sima, A. F., and T. Brismar (1985) Reversible diabetic nerve dysfunction: Structural correlates to electrophysiological abnormalities. *Ann. Neurol.* 18: 21–29.
- Smith, K. J., and C. L. Schaaf (1981) Size-dependent variation of nodal properties in myelinated nerve. *Nature* 293: 297–299.
- Stampfli, R., and B. Hille (1976) Electrophysiology of frog peripheral myelinated nerve. In *Neurobiology of the Frog*, R. Llinás and W. Precht, eds., Springer-Verlag, New York.
- Swadlow, H. A., and S. G. Waxman (1978) Activity dependent variations in the conduction properties of central axons. In *Physiology and Pathobiology of Axons*, S. G. Waxman, ed., Raven, New York.
- Tasaki, I. (1939) The strength duration relation of the normal polarized and narcotized fiber. *Annu. J. Physiol.* 125: 367–379.
- Tasaki, I. (1953) *Nervous Transmission*, Charles C Thomas, Springfield, IL.
- Villegas, J. (1972) Axon-Schwann cell interaction in the squid nerve fiber. *J. Physiol. (Lond.)* 225: 275–296.
- Wiley, C. A., and M. E. Ellisman (1980) Rows of dimeric particles within the axolemma and juxtaposed particles within glia, incorporated into a new model for the paranodal glial-axonal junction at the node of Ranvier. *J. Cell Biol.* 84: 261–280.
- Williams, P. L., and D. N. Landon (1963) Paranodal apparatus of peripheral nerve fibers of mammals. *Nature* 198: 670–673.
- Wurtz, C. C., and M. H. Ellisman (1985) Effects of specimen preparation on activity associated changes in node of Ranvier structure. *Am. Soc. Cell Biol.* 101: 124a.
- Zachar, J. (1967) Electrophysiology of peripheral excitable structures *in situ*. In *Electrophysiological Methods in Biological Research*, 3rd ed., J. Bures, M. Petran, and J. Zachar, eds., Academic, New York.
- Zagoren, J. C., and J. C. Arezzo (1982) Cation binding at the node of Ranvier: II. Redistribution of binding sites during electrical stimulation. *Brain Res.* 242: 27–31.