MICROTUBULE-NEUROFILAMENT SEGREGATION PRODUCED BY β,β' -IMINODIPROPIONITRILE: EVIDENCE FOR THE ASSOCIATION OF FAST AXONAL TRANSPORT WITH MICROTUBULES¹

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Abstract

The subperineurial injection of β,β' -iminodipropionitrile (IDPN) into rat sciatic nerves resulted in focal disorganization of the axonal cytoskeleton characterized by segregation of neurofilaments and microtubules. Shortly after injection, microtubules clustered together to form a central channel, while neurofilaments became chaotically arrayed between the microtubule channel and axolemma. Electron microscopic autoradiography disclosed that rapidly transported organelles were preferentially associated with the microtubule-enriched central channels. These studies indicate that IDPN acts at the level of the axon to disrupt interactions between cytoskeletal elements and show that rapidly transported constituents are preferentially conveyed in association with microtubules. The model provides an opportunity to dissect the interactions of the cytoskeletal elements and other organelles.

The polypeptides comprising the neurofilaments and microtubules of the axonal cytoskeleton are conveyed down the axon by slow axonal transport (Hoffman and Lasek, 1975; Lasek and Hoffman, 1976). In contrast, particulate organelles are carried primarily by fast transport. In the anterograde direction, these organelles include smooth membrane-bound vesicles (Tsukita and Ishikawa, 1980) and/or tubules (Droz et al., 1973, 1975; Rambourg and Droz, 1980), while among organelles undergoing retrograde transport, larger vesicular structures and dense bodies predominate (LaVail and LaVail, 1974; Tsukita and Ishikawa, 1980).

These rapidly transported organelles must pass through the cytoskeleton, and models based on interactions between cytoskeletal elements and rapidly moving constituents have been widely suggested (for reviews, see Grafstein and Forman, 1981; Ochs, 1981). In particular, several lines of evidence suggest a role for microtubules

in fast axonal transport: fast transport is blocked by agents that impair polymerization of tubulin or promote the disassembly of microtubules (Kreutzberg, 1969; Dahlstrom, 1971; Schlaepfer, 1971; Ghetti and Ochs, 1978; Samson et al., 1979), and rapidly transported organelles frequently show spatial associations with microtubules (Raine et al., 1971; Tsukita and Ishikawa, 1981). However, it has been difficult to test critically the hypothesis that rapidly transported organelles are specifically associated with microtubules, because other cytoskeletal elements are also arrayed near, and interact with, the microtubules. Indeed, the concept that transport mechanisms are independent of microtubules has received some experimental support (Byers, 1974; Brady et al., 1980).

One way of looking at the role of cytoskeletal components in transport processes is to perturb the cytoskeleton and then examine the relationship between rapidly transported elements and the individual cytoskeletal organelles. Papasozomenos et al. (1981) have reported that systemic administration of β,β' -iminodipropionitrile (IDPN) produces a distinctive reorganization of the axonal cytoskeleton in which neurofilaments form a subaxolemmal ring surrounding a central region enriched in microtubules. The present study was designed to determine if IDPN, focally applied along the course of nerve fibers, could produce local changes in the organization of the axonal cytoskeleton similar to those produced by systemic administration, and, if so, whether rapidly trans-

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ported organelles might be preferentially associated with specific cytoskeletal elements.

Materials and Methods

Agents injected

IDPN, received as a clear, colorless liquid from Eastman (Rochester, NY; lot number 6555) was diluted 1:1 with Ringer's solution and brought to pH 7.3. Control solutions included Ringer's solution, normal rat serum, sucrose (4.0 M), β -aminopropionitrile (BAPN) (4.0 M), and propionitrile (PN) (4.0 M). The solution to be tested was drawn into a micropipette (30 to 40 μ m tip diameter) connected by a mineral oil-filled plastic tube to a Hamilton syringe fixed in a variable speed infusion pump.

Subperineurial injections

Male Sprague-Dawley rats (350 gm) were anesthetized with chloral hydrate (400 mg/kg), and the sciatic nerves were exposed in the upper thigh. Just below the branching of the nerve to the biceps femoris, the loose connective tissue overlying the sciatic nerve was removed, and a small portion of the perineurium was gripped with a watchmaker's forceps. A 34 gauge needle was used to penetrate the perineurium, and the micropipette, mounted on a micromanipulator, was advanced through the perineurial puncture in a direction nearly parallel to the nerve so that the pipette tip lay just beneath the perineurium. Ten microliters of the solution were injected into the endoneurial compartment over a 5-min period. In most animals, IDPN was injected into one nerve, and the contralateral sciatic nerve was injected with one of the control solutions.

Fixation and tissue preparation

Two to 24 hr after injection, nerves were re-exposed, and the nerve bed was flooded with 5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.3). After 10 min of *in situ* fixation, the nerves were removed, fixed for an additional period, and divided into 5-mm segments. The segments of nerve containing the injection site and the segments immediately proximal and distal to the site were removed, gently agitated in 2% osmium tetroxide buffer for 2 hrs, dehydrated in graded alcohols, and embedded in Epon/Araldite. Thick sections (1 μ m) were stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate.

Labeling of axonal transport

Animals were labeled by techniques described elsewhere (Griffin et al., 1976, 1981). [^{3} H]Fucose (New England Nuclear) (60 Ci/mmol) was blown to dryness under a stream of nitrogen and restored to a concentration of 50 μ Ci/ μ l in Ringer's solution. One microliter of this solution was slowly injected into each of eight sites in the lumbar ventral horns of rats, using a micromanipulator-mounted micropipette connected to tubing and a syringe as described above. After injection, the rectal temperature was regulated to 37°C.

Autoradiographic studies

Following injection of [^{3} H]fucose, while the animals were still anesthetized, a test solution was injected into

one sciatic nerve as described above; this solution was IDPN in two nerves and sucrose in two others. Seven or 8 hr later, the animals were heparinized and perfused through the ascending aorta with saline followed by 5% glutaraldehyde. The injected segment, as well as the segments immediately proximal and distal, were removed. Segments were also taken from the same regions of the untreated contralateral nerves. All samples were embedded and prepared for EM autoradiography as previously described (Griffin et al., 1981). Briefly, pale gold sections were dipped in Ilford L4 emulsion, exposed for 12 or 33 weeks, and then developed in Kodak D19 developer. To minimize overlapping of silver grains, only 12-week exposures were quantitatively analyzed. The specimens were photographed and the grain distribution was determined from prints (final magnification $\times$ 30,000) by established methods (Salpeter et al., 1969). To assess the relation of grain distribution to the microtubule-rich central channels and other compartments (see below), fibers with well defined channels were selected, and the channels were outlined. A series of concentric rings was drawn outside (and inside) the outline of the channel with each subsequent ring larger (or smaller) in radius than the previous one by one half-distance unit (one half-distance (HD) unit = 1600 Å) (Salpeter et al., 1969). The area of each resulting band (i.e., the area between two adjacent rings) was measured using a Hewlett-Packard 9864 digitizer coupled to a 9815S programmable calculator. Each silver grain was circumscribed by the smallest possible circle, and the number of grain circles whose center lay in each band was tabulated. The results, expressed as grain densities (grains/ μ m²) were normalized and plotted. To provide a comparison between the experimental and control nerves (which had no central channels), the analysis was repeated beginning with the band centered on the axolemma; these results were then analyzed and plotted as previously described (Griffin et al., 1981).

Results

Morphological changes

Nerves were examined 2 to 24 hr after endoneurial injection of IDPN. Cytoskeletal changes were confined to the injected segment (approximately 5 mm in length); they were not present in more proximal or distal segments of the nerve. The specificity of these local changes for IDPN was established by examination of control nerves which had been injected with sucrose, Ringer's solution, serum, BAPN, or PN solutions. In control nerves, fibers were usually normal (Fig. 1A). Nonspecific changes were seen in all injected nerves and included some increase in separation of the endoneurial elements (presumably due to increase in endoneurial fluid) and evidence of a few transected axons. A few nerves showed severe swelling of Schwann cell cytoplasm and inner Schwann cell collars. When either control or IDPN-injected nerves showed this change, the tissues were discarded.

In 27 successful preparations from IDPN-treated nerves, morphological changes were essentially restricted to the axon. The characteristic finding, a "bull's-eye" of dark granular material in the center of the fiber sur-

Figure 1. *A* and *B*, Light micrographs of transverse sections of rat sciatic nerve injected subperineurially with sucrose solution (*A*) or IDPN (*B*). In *A*, the appearance of the nerve fibers is normal; in *B*, the nerve fibers contain dark granular material usually located in the central region of the fiber. One-micrometer sections stained with toluidine blue. Magnification $\times 1,350$. *C*, Electron micrograph of a transverse section of nerve fiber 24 hr after subperineurial injection of IDPN. Note that a sharply demarcated central channel of microtubules (outlined by arrows) is present in the center of the fiber and that most mitochondria and smooth membrane-bound profiles are found in relation to that channel. The surrounding neurofilaments are chaotically organized and randomly oriented with regard to the long axis of the fiber. The boxed region is shown in Figure 2*D*. Magnification $\times 6,500$. *D*, Higher magnification shows the margin between the central channel and the surrounding neurofilamentous zone. The few neurofilaments remaining near the central channel maintain their longitudinal orientation. Note the arrangement of microtubules around mitochondria (arrows) and the persistence of the cross-bridges between adjacent microtubules. *MT*, microtubules; *NF*, neurofilaments. Magnification $\times 57,000$.

rounded by a pale glassy appearing ring (Fig. 1*B*), could be appreciated at the light microscopic level. These central or paracentral targets were present by 6 hr after endoneurial injection of IDPN. Electron microscopy disclosed that these targets resulted from the clustering of mitochondria (Fig. 1*C*); these clusters were, in turn, regularly associated with an alteration of the axonal cytoskeleton in which microtubules collected within a central channel, while neurofilaments were arrayed in a

surrounding circumferential ring between the channel and the axolemma (Figs. 1, *C* and *D*, and 2).

The temporal and spatial evolution of these changes was reconstructed by examining axons at various time intervals after injection. By 2 hr, the microtubules were gathering in the center of the fiber, but the channels were usually not well defined (Fig. 2). Neurofilaments tended to be located in the subaxolemmal region but, at this stage, they retained their normal predominantly longi-

Figure 2. Electron micrograph of a transverse section of myelinated nerve fiber 2 hr after subperineurial injection of IDPN. The microtubules are beginning to collect in the center of the fiber. The neurofilaments remain oriented predominantly in the long axis of the fiber and some remain within the central region, although a tendency toward subaxolemmal distribution is already apparent. Mitochondria and smooth membrane-bound profiles are more frequently seen in the center of the fiber than in the surrounding subaxolemmal zone. Both the Schwann cell and myelin sheath remain normal. Magnification $\times 11,000$.

tudinal orientation. Some neurofilaments were present within the center of the fiber, and some clusters of microtubules remained within the subaxolemmal ring. Between 2 and 24 hr, the central channels became progressively well demarcated (Figs. 1*B* and 3). In many fibers, neurofilaments were almost completely excluded from the central channels (Fig. 1). The neurofilaments became progressively more disoriented with time. The change in orientation of neurofilaments began peripherally and appeared to advance toward the center of the fiber (Fig. 3). By 6 hr, virtually all neurofilaments in many fibers were arranged chaotically (Figs. 1 and 3). Even at this stage, neurofilament sidearms were apparent, but no judgment could be made with regard to the extent of actual cross-bridging because of the altered orientation of the neurofilaments with respect to each other (Fig. 3*C*). In some fibers, granular and amorphous material appeared within the filamentous ring; this material occasionally appeared to replace the neurofilaments, suggesting that it might be derived from disassembly or degradation of neurofilaments.

In association with changes in the cytoskeleton, the distribution of particulate organelles within the IDPN-treated fibers was dramatically altered. As indicated above, mitochondria were predominantly and often exclusively associated with the central channels (Figs. 1 and 3). Smooth membrane-bound profiles were found within both the central channels and immediately beneath the axolemma (Figs. 1 and 2). Definitive classifications of these membranous profiles were not attempted on the basis of these routine thin sections. In a small number of fibers, the amounts of smooth membrane-bound organelles were increased. Massive accumulations of particulate organelles, such as occur with axonal inter-

ruption (Zelena et al., 1968; Griffin et al., 1977) or treatment with agents which impede fast transport (Schlaepfer, 1971; Tsukita and Ishikawa, 1980), were uncommon and were present just beneath the perineurium in proximity to the immediate site of injection; these changes were interpreted to be a consequence of injection trauma or excessively high local concentrations of IDPN.

Within the central channels, clusters of microtubules were present around mitochondria and elements of smooth membrane-bound profiles (Fig. 1). Cross-bridges, apparently extending from microtubules to microtubules, neurofilaments, or mitochondria and to membrane-bound profiles, were readily identified (Figs. 1*D* and 3*B*).

Passage of fast axonal transport through the injected segments

EM autoradiographic studies. To examine the distribution of rapidly transported organelles as they passed through areas of cytoskeletal segregation, [^3H]fucose was injected into the spinal cords of animals, and IDPN was injected subperineurially into the sciatic nerve immediately afterward. The animals were then killed at 7 or 8 hr, and the nerves were removed and prepared for autoradiography. At this time, the peak of fast transport was within the injected segment.

In control nerves and in regions of IDPN-treated nerves in which cytoskeletal segregation was not seen, silver grains were distributed throughout the axon including both subaxolemmal and central regions (Fig. 4). In the fibers with cytoskeletal segregation, the rapidly transported elements were prominently associated with the microtubule channels (Fig. 5). Quantitative analysis of grain distribution confirmed this association (Fig. 6).

Figure 3. Electron micrographs of longitudinal sections of nerves 6 hr after subperineurial injection of IDPN. **A**, Two fibers, illustrating central channels, extend over long lengths of the fibers. In the *lower fiber*, few neurofilaments remain in the central channel. Surrounding neurofilaments maintain a predominantly longitudinal orientation, but malorientation of neurofilaments is apparent just beneath the axolemma in some regions. In the *upper fiber*, the neurofilaments are chaotically organized except near the central channel. Magnification $\times 4,200$. **B**, This fiber shows the interface between longitudinally oriented neurofilaments within the central channel and the chaotically oriented neurofilament outside the channel. *MT*, microtubules; *NF*, neurofilaments. Magnification $\times 19,700$. **C**, Higher magnification of a region in which neurofilaments remain within the central channel. Note that cross-bridges can be seen between microtubules and microtubules (*arrows*), between microtubules and neurofilaments (*upper box*), and between neurofilaments and adjacent neurofilaments (*lower box*). Magnification $\times 48,000$.

In those fibers where clusters of grains were found remote from the central channels, microtubules could often be identified within the labeled regions. Grains were infrequent in regions devoid of microtubules. The quantitative analysis did not disclose preferential labeling in the axolemmal or immediate subaxolemmal regions (Fig. 6*B*).

Discussion

Papasozomenos et al. (1981) have recently described reorganization of the axonal cytoskeleton following systemic administration of IDPN. Our results extend these observations and demonstrate that local application of

Figure 4. EM autoradiographs showing the distribution of radioactivity in control nerve fibers (without cytoskeletal segregation) 8 hr after intraspinal injection of [^{3} H]fucose and endoneurial injection of sucrose just below the branching of the nerve to the biceps femoris. Note the silver grains distributed widely throughout the axons. **A**, Exposure, 12 weeks. Magnification $\times 4,500$. **B**, Exposure, 33 weeks. Magnification $\times 23,000$.

IDPN along the course of a nerve fiber initiates a rapidly evolving focal disorganization of the cytoskeleton. These changes result in segregation of microtubules into central channels surrounded by a subaxolemmal ring of neurofilaments. The sequence of changes can be summarized as follows: by 2 hr, microtubules begin to collect in poorly demarcated central channels; the surrounding neurofilaments remain longitudinally oriented. At this stage, the fibers resemble those seen in a few days after systemic administration of IDPN (Papsozomenos et al., 1981). By 6 hr, the microtubule-rich channels become consolidated, often resulting in complete exclusion of neurofilaments.

Relegated to the periphery, neurofilaments lose their longitudinal direction and become chaotically arranged. We have described the subsequent evolution of this alteration separately (J. W. Griffin, K. E. Fahnestock, and D. L. Price, submitted for publication). By 24 hr, the cytoskeletal segregation is no longer seen in a substantial proportion of fibers, suggesting that the lesion is reversible. By this time, some fibers have developed increased density of neurofilaments and focal neurofilamentous axonal swellings in and proximal to the site of injection, reproducing the neurofibrillary pathology induced by systemic administration (Chou and Hartmann, 1964,

Figure 5. Transverse sections of an EM autoradiogram from a nerve 8 hr after intraspinal injection of [^3H]fucose and endoneurial injection of IDPN just below the branching of the nerve to the biceps femoris. Note silver grains concentrated over the central microtubule channels. Some fibers also appear to have grains near the axolemma. Grains are uncommon in the neurofilamentous zone. *A*, Exposure, 12 weeks. Magnification $\times 4,000$. *B*, Exposure, 33 weeks. Magnification $\times 26,000$.

1965; Griffin and Price, 1980, 1981a, b). Although some fibers undergo Wallerian degeneration, this does not appear to be a necessary outcome of either cytoskeletal segregation or formation of neurofilamentous swellings, since many nerves show little evidence of Wallerian degeneration at 3 to 7 days after endoneurial injection.

The present study focuses on the passage of rapidly transported organelles through regions of early cytoskeletal segregation (before neurofilamentous accumulations are prominent). This preparation provides an excellent opportunity to study the relationship of fast

transport to specific cytoskeletal elements and other axonal constituents. Unlike many other agents that perturb the axonal cytoskeleton, such as colchicine (Kreutzberg, 1969; Dahlstrom, 1971), vincristine (Schlaepfer, 1971), and the maytansinoids (Ghetti and Ochs, 1978), IDPN, as used in this study, does not block fast transport. The segregation of microtubules from both neurofilaments and axolemma is sufficiently well demarcated to allow assessment of the association of transported organelles with these elements.

The EM autoradiographic observations clearly dem-

Figure 6. Histograms demonstrating the distribution of silver grains in EM autoradiograms of control (A) and IDPN-injected fibers (B and C). In both A and B, the axolemma is at 0 with positive HD units inside and negative HD units outside the axon (within the myelin sheath). In C, a ring was drawn around the segregated microtubule/mitochondria channel which is represented by 0 on the graph. +HD units are outside the channel, and -HD units are inside.

onstrate that, in IDPN-injected nerve segments, rapidly transported elements move in association with the microtubule channels. This observation appears incompatible with three previous hypotheses: that neurofilaments might act as a substrate for fast transport (Hoffman and Lasek, 1975; Willard and Simon, 1981); that fast transport might depend upon an actin-myosin system (LeBeux and Willemot, 1975; Isenberg et al., 1980) located immediately beneath the axolemma; and that fast transport may, in fact, represent intra-axolemmal migration of proteins (Marchisio et al., 1975). With regard to the last two possibilities, it is noteworthy that we visualized only small amounts of label in the axolemmal or immediate subaxolemmal regions (Fig. 6); although these regions occasionally appeared more heavily labeled than the neurofilament zone (Fig. 5B), there was no quantitative

evidence for a selective grain distribution in the subaxolemmal zone (Fig. 6). These observations argue strongly against neurofilaments, subaxolemmal contractile systems, or intra-axolemmal migration of proteins being obligatory pathways for the movement of substantial fractions of rapidly transported materials.

The present observations demonstrate a relationship between microtubules and fast transport but provide no data regarding the precise role played by the microtubules. Our results are compatible with models of fast transport which invoke obligatory participation by microtubules in force generation or translocation of membranous elements (Ochs, 1981). However, it is also possible that microtubules provide the preferred cytoskeletal framework for a nonmicrotubule-dependent motile system (Edds, 1975).

The present results clearly demonstrate that local application of IDPN is sufficient to induce two characteristic pathological features: the dissociation of microtubules from neurofilaments with the formation of channels (Papazosomenos et al., 1981; Griffin et al., 1982; J. W. Griffin, K. E. Fahnestock, and D. L. Price, submitted for publication) and the subsequent accumulation of neurofilaments (Chou and Hartmann, 1964, 1965; Clark et al., 1980; Griffin and Price, 1980, 1981a, b). These observations suggest two conclusions with regard to the pathogenesis of lesions following systemic administration of IDPN. First, a direct action of the agent upon the axon (rather than the cell body) is sufficient to induce the neurofibrillary pathology. Thus, the disorder is one of a growing number of neuropathies in which the designation "toxic axonopathy" is both pathologically and pathogenetically correct (Glazer et al., 1979; Griffin and Price, 1980; Politis et al., 1980; Spencer and Schaumburg, 1980). Second, it is likely that the cytoskeletal segregation following local or systemic administration of IDPN (Papazosomenos et al., 1981; Griffin et al., 1982) underlies the defect in axonal transport. Previous studies (Griffin et al., 1978; Yokoyama et al., 1980) have shown that systemic administration of IDPN impairs slow axonal transport in large fibers, most severely retarding movement of the neurofilament proteins.

On the basis of this and previous studies, we can tentatively reconstruct the pathogenesis of IDPN-induced axonopathy, as follows. High concentrations of IDPN promptly initiate a disorganization of the cytoskeleton, dissociating neurofilaments from microtubules. The mechanism underlying this effect is not known, but it may involve disruption or incompetency of the cross-bridges between neurofilaments and microtubules. The microtubules accumulate in the central regions of the nerve fiber; many of the particulate organelles within the axon, including mitochondria, also collect within this region. Within the central channel, fast axonal transport is maintained. In the surrounding zone, neurofilaments initially maintain their longitudinal orientation but, in time, become markedly maloriented. The movement of neurofilaments is severely impaired and, as more neurofilaments are delivered to the area, the density of neurofilaments increases. As neurofilaments accumulate, the axon begins to enlarge and eventually becomes markedly swollen (J. W. Griffin, K. E. Fahnestock, and D. L. Price, submitted for publication). In the case of locally injected nerves, the accumulation of neurofilaments represents those moving into the injection site from more proximal segments. In systemically intoxicated animals, neurofilaments tend to occur in the proximal regions, because the cell body continues to synthesize neurofilaments and deliver them to axons incapable of transporting these polypeptides beyond the proximal regions (Griffin et al., 1978; Yokoyama et al., 1980).

The ability to reproduce major features of systemic intoxication by local administration of IDPN provides an excellent preparation for biochemical analyses of the action of this agent. The use of IDPN in this and other systems offers an opportunity for future exploration of the factors normally responsible for assembly, organization, maintenance, and transport of the axonal cytoskeleton.

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