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Organization and Slow Axonal Transport of Cytoskeletal Proteins Under Normal and Regenerating Conditions

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Abstract

The organization of the axonal cytoskeleton was investigated by analyzing the solubility and transport profile of the major cytoskeletal proteins in motor axons of the rat sciatic nerve under normal and regenerating conditions. When extracted with the Triton-containing buffer at low temperature, 50% of tubulin and 30% of actin were recovered in the insoluble form resistant to further depolymerizing treatments. Most of this cold-insoluble form was transported in slow component a (SCa), the slower of the two subcomponents of slow axonal transport, whereas the cold-soluble form showed a biphasic distribution between SCa and SCb (slow component b).

Changes in slow transport during regeneration were studied by injuring the nerve either prior to (experiment I) or after (experiment II) radioactive labeling. In experiment I where the transport of proteins synthesized in response to injury was examined, selective acceleration of SCb was detected together with an increase in the

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relative proportion of this component. In experiment II where the response of the preexisting cytoskeleton was examined, a shift from SCa to SCb of the cold-soluble form was observed. The differential distribution and response of the two forms of tubulin and actin suggest that the cold-soluble form may be more directly involved in axonal transport.

Index Entries: Axonal transport; nerve regeneration; cytoskeletal proteins; tubulin; actin.

Introduction

Much of the information concerning composition of the axonal cytoskeleton has been gained from studies on slow axonal transport, which is a flow of cytoskeletal proteins down the axon (Lasek and Hoffman, 1976; Willard and Hulebak, 1977; Black and Lasek, 1980; Levine and Willard, 1980; Tytell et al., 1981). In this article, we describe attempts to investigate the dynamic organization of the axonal cytoskeleton through biochemical analyses of solubility and transport profile of cytoskeletal proteins in the normal and the regenerating nerve.

One of the characteristic features of the axonal cytoskeleton is its stability against depolymerization, detected morphologically, on one hand, as stable fragments of microtubules (Heidemann et al., 1984; Sahenk and Brady, 1987; Baas and Black, 1990) and biochemically, on the other, as insoluble tubulin and actin (Black et al., 1984; Brady et al., 1984; Tashiro et al., 1984; Tashiro and Komiya, 1987,1989). In the first part of the article, we show how such insoluble tubulin and actin are related to the two subcomponents of slow axonal transport, slow component a (SCa or group V) and slow component b (SCb or group IV), which differ in rates and compositions (Willard and Hulebak, 1977; Black and Lasek, 1980; Levine and Willard, 1980; Tytell et al., 1981).

Following nerve injury, large qualitative and quantitative changes in cytoskeletal protein synthesis are known to take place in the cell body (for review, *see* Bisby and Tetzlaff, 1992), resulting in changes in the rate and composition of slow axonal transport (Grafstein and Murray, 1969; Hoffman and Lasek, 1980; Hoffman et al., 1985b; Oblinger and Lasek, 1988). In addition to this altered synthesis, reorganization of the preexisting cytoskeleton must also take place since supply of the new set of cytoskeletal proteins synthesized in response to regeneration is not fast enough to reach the injury site in time to form the growth cone and support initial sprouting (Komiya, 1981b; McQuarrie and Lasek, 1989). In the present article, these two types of reactions are distinguished by altering the order of nerve injury and radioactive labeling. The results indicate that, in response to nerve injury, significant reorganization takes place both in the newly synthesized and the preexisting cytoskeleton already in transit (Tashiro and Komiya, 1991a).

Methods

Radioactive Labeling and Axonal Transport of Cytoskeletal Proteins

Male Wistar rats, 7 wk old at the time of radioactive labeling, were used. Under ether anesthesia, L-[^{35}S]methionine (>1000 Ci/mmol; New England Nuclear, Boston) concentrated by lyophilization (25 μCi in 0.2 μL) was injected into the anterior horn area of L_3–L_5 spinal cord twice on each side. Two or 3 wk after injection, sciatic nerve and ventral root were dissected out, frozen on a plastic plate, and cut into 6-mm consecutive segments. The segments were kept at –80°C until use.

Nerve Injury and Regeneration

Male Wistar rats, 5, 6, or 7 wk old, were used according to the two types of experimental protocols shown in Fig. 1, so that all of them received radioactive methionine at 7 wk of age.

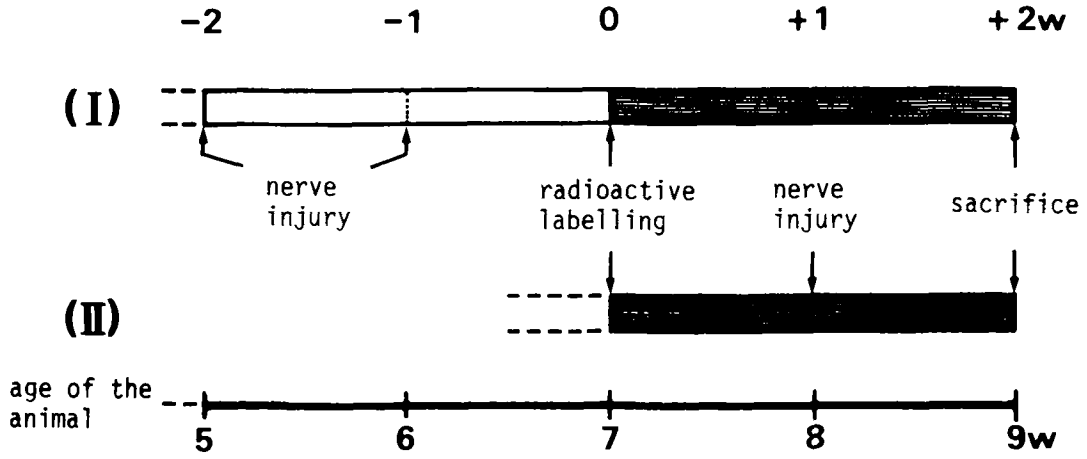

Fig. 1. Protocols for the two types of regeneration experiments. In experiment I, changes in axonal transport of cytoskeletal proteins synthesized in response to nerve injury are examined. In experiment II, the effect of injury on the preexisting cytoskeletal proteins already in transit is observed.

With the animal under ether anesthesia, the sciatic nerve was exposed at the midthigh level (70–80 mm from the spinal cord) and frozen by pressing against it a piece of copper wire precooled in liquid nitrogen, twice for 10 s each, on both sides. The axotomy was highly reproducible and complete by this procedure. The initial lag before the onset of active regeneration is minimal (1 d) compared with other methods, such as crush or transection with suture (Forman and Berenberg, 1978; Forman et al., 1979; Komiya, 1981a). Radioactive labeling was carried out as described above when the pretreated (experiment I) or untreated (experiment II) animals were 7 wk old to avoid age-dependent variations in transport (Komiya, 1980; Hoffman et al., 1985a; McQuarrie et al., 1989; Tashiro and Komiya, 1991b).

Fractionation of Transported Proteins

For each time-point in each type of experiment, two to three animals were used. For each measurement, a pair of nerves from one animal was processed together. A pair of 6-mm nerve segments from identical positions was frozen in liquid nitrogen and crushed to fine powder in a stainless-steel mortar with a hammer operated by compressed air and homogenized in 2.5 mL of ice-cold Triton-buffer containing 1% Triton X-100, 50 m*M* Tris (pH 7.5), 25 m*M* KCl, 1 m*M* $MgCl_2$, 5 m*M* EGTA, 5 m*M* dithiothreitol (DTT), and 0.25 m*M* phenylmethylsulphonyl fluoride (PMSF). The homogenate was layered onto 3 mL of Triton-buffer containing 0.25*M* sucrose in addition and centrifuged at 100,000*g* for 1 h. Triton-soluble proteins in the supernatant were precipitated with 10% trichloroacetic acid and washed twice with ethanol.

Labeled proteins in both the Triton-soluble and -insoluble fractions were separated either by SDS-PAGE (Laemmli, 1970) or two-dimensional gel electrophoresis (O'Farrell, 1975), and visualized by fluorography (Bonner and Laskey, 1974). For the measurement of radioactivity, SDS-PAGE gels were stained with Coomassie Brilliant Blue R-250 after electrophoresis, and the bands corresponding to tubulin, actin, and the three neurofilament subunits were cut out. Radioactivity in each gel piece was determined by liquid scintillation counting following overnight extraction with Soluene 350 (Packard Instrument, Downers Grove, IL).

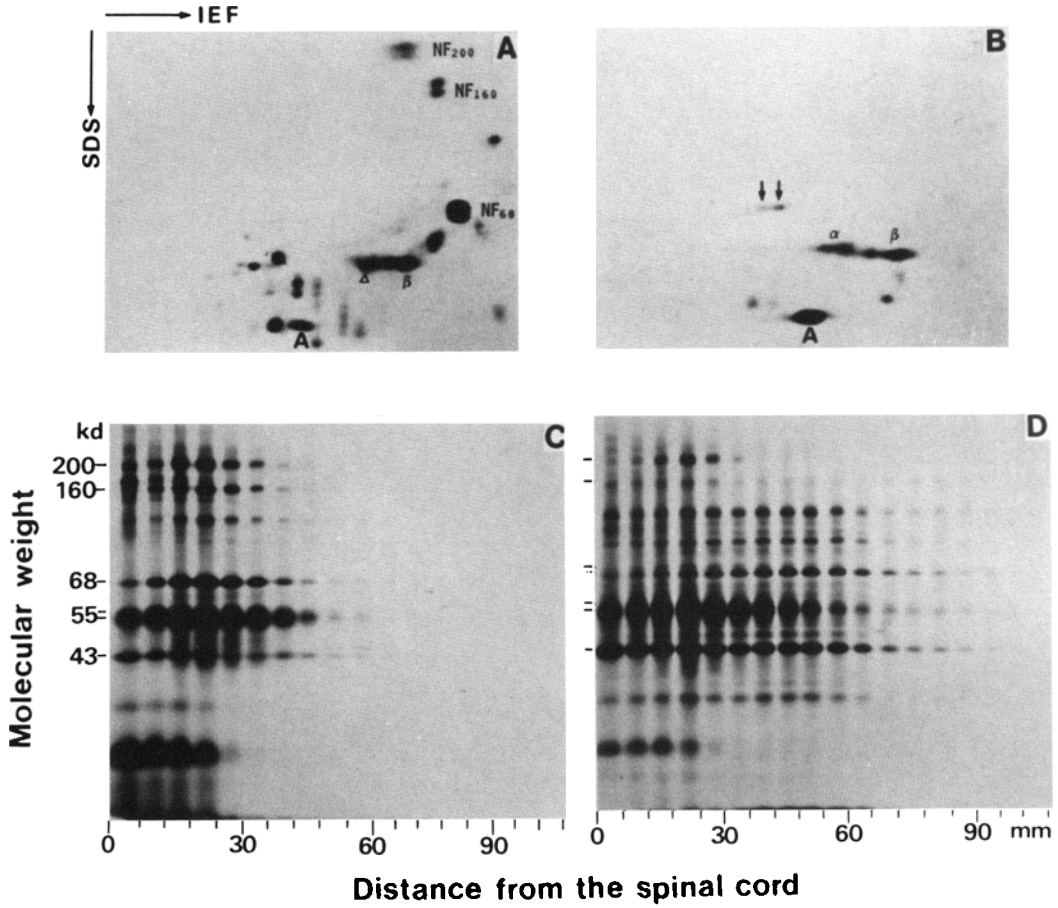

Fig. 2. Soluble and insoluble forms of cytoskeletal proteins in slow axonal transport. Labeled proteins in 6-mm segments of the rat sciatic nerve obtained 2 wk after radioactive labeling of the spinal cord were fractionated into insoluble (A,C) and soluble (B,D) forms by extraction with Triton buffer at low temperature as described in Methods. In A and B, proteins in the pooled 10 segments were fractionated together, separated by two-dimensional electrophoresis, and visualized by fluorography. Although tubulin and actin were found both in (A) insoluble and (B) soluble fractions, the insoluble fraction (A) lacked the usual set of α- and β-tubulins found in the soluble (B) fraction. Instead, a modified α-tubulin with higher mobility in SDS is detected (Δ in A). The 68-kDa protein in (B) is not NF68 (↓ in B). NF_{200}, NF_{160}, NF_{68}: the three neurofilament subunits. α: α–tubulin. β: tubulin. β–tubulin. In (C) and (D), insoluble (C) and soluble (D) proteins contained in each pair of 6-mm segments at identical positions were separated by SDS PAGE and visualized by fluorography. Positions of tubulin (55 kDa), actin (43 kDa), and the neurofilament proteins (200, 160, 68 kDa) are marked with their molecular masses.

Results

Cold-Soluble and Cold-Insoluble Forms of Tubulin and Actin Transported in the Axon

As shown in Fig. 2, 50% of labeled tubulin transported in the sciatic nerve 2 wk after radioactive labeling of the spinal cord was found to be insoluble in 1% Triton-containing buffer at low temperature, a condition that depolymerizes cytoplasmic microtubules. The amount of cold-insoluble tubulin ranged from 30 to 70%, depending on the age of the animal and the duration of transport (Tashiro and Komiya, 1989;1991b). Further treatment with other microtubule-destabilizing agents such as Ca^{2+}, colchicine, and nocodazole did not solubilize the cold-insoluble

tubulin, suggesting that it comprised a distinct population (Tashiro and Komiya, 1989). On two-dimensional electrophoresis, it was shown that the insoluble tubulin lacked the normal set of α-tubulins detected in the soluble fraction or in cycled microtubules obtained from the brain, but was enriched in the α-tubulin isotype with higher electrophoretic mobility in SDS (Δ in Fig. 2A; Tashiro and Komiya, 1983). About 30% of labeled actin was also recovered in the cold-insoluble fraction, which was not solubilized by further homogenization and dilution in low-ionic-strength solution.

Most of the neurofilament proteins were in the insoluble fraction, except for some of the 200-kDa subunit (NF200). The 68-kDa component detected in the cold-soluble fraction (↓ in Fig. 2B) was not the 68-kDa neurofilament subunit (NF68). It had much higher isoelectric point (p*I* 6.0) compared with that of NF68 (p*I* 5.3) as shown in Fig. 2B. Further analysis revealed that it was composed of two different proteins, the 70-kDa constitutively synthesized heat-shock protein (HSC 70: de Waegh and Brady, 1989; Sekimoto et al., 1991) and the Ca^{2+}-dependent lipid-binding protein annexin VI (Sekimoto et al., 1991).

When labeled proteins in each consecutive 6-mm segment of the nerve were separated into soluble and insoluble fractions as above and analyzed, it became evident that tubulin and actin in the cold-soluble fraction migrated farther distally ahead of those in the cold-insoluble fraction (Fig. 2C,D).

For a more detailed analysis, radioactivity associated with tubulin, actin, and neurofilament protein bands in each column of SDS gels, such as those in Fig. 2C and D, was measured separately, and radioactivity distribution along the nerve was expressed as percentage of total tubulin- (Fig. 3A,B) or actin-associated (Fig. 3D,E) radioactivity. The transport profiles thus obtained clearly demonstrated the presence of two rate components (Fig. 3). In the case of tubulin, a shoulder with higher soluble-to-insoluble ratio (42–66 mm from the spinal cord) was observed toward the migrating front of radioactivity at 2 wk postlabeling (Fig. 3A,C), which became separated into a faster wave at 3 wk (Fig. 3B). In the case of actin, the presence of a faster migrating component enriched in soluble actin was already evident at 2 wk postlabeling (Fig. 3D), since the proportion of radioactivity in this component was much higher than that for tubulin. The location of the faster actin wave as well as the slower main wave agreed well with those of tubulin at both 2 and 3 wk postlabeling. Average rates of transport for the two waves in both cases were 1.5 and 3 mm/d, respectively (rates of migration of the peaks of radioactivity). Neurofilament proteins were confined to the slower main wave as represented by the transport profile for NF68 in Fig. 3A and B.

From the presence of neurofilament proteins, the slower wave seems to correspond to SCa or group V originally defined in the optic system as the slower of the two subcomponents of slow axonal transport (Lasek and Hoffman, 1976; Willard and Hulebak, 1977; Black and Lasek, 1980; Levine and Willard, 1980). The faster wave corresponds to SCb or group IV. In contrast to the situation found in the optic system, where tubulin was localized to SCa and actin to SCb, tubulin and actin in the sciatic nerve were present both in SCa and SCb (Tashiro et al., 1984; McQuarrie et al., 1986; Oblinger et al., 1987; Tashiro and Komiya, 1987,1989). The ratio of cold-soluble to cold-insoluble tubulin or actin in each segment (Fig. 3C,F) clearly indicates that major differences in solubility properties exist between these cytoskeletal proteins contained in SCa and SCb.

Solubility and Transport Profile of Cytoskeletal Proteins in the Regenerating Nerve

Two types of experiments were carried out in order to detect changes in organization and transport of cytoskeletal proteins during regeneration (Fig. 1). In experiment I, nerves were injured 1 or 2 wk prior to radioactive labeling of the spinal cord to observe axonal transport of cytoskeletal proteins newly synthesized during regeneration.

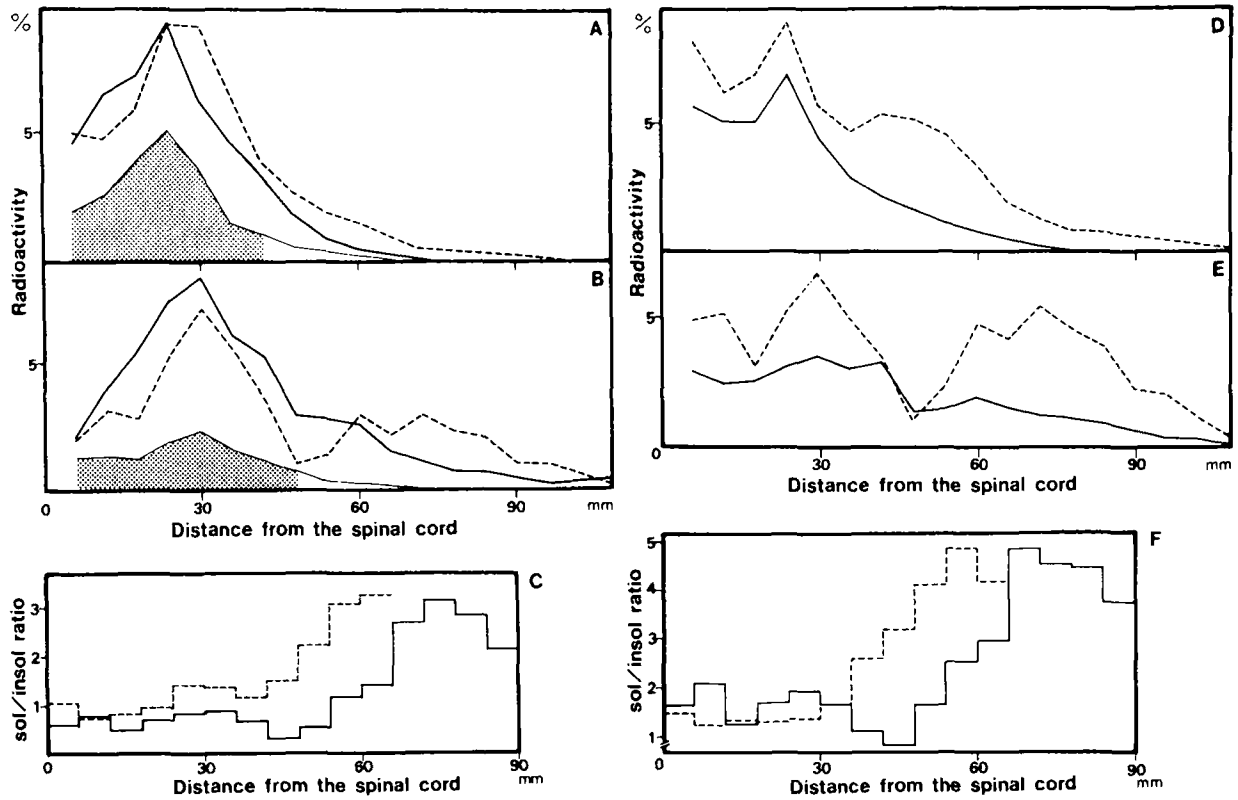

Fig. 3. Axonal transport of soluble and insoluble tubulin (A,B,C) and actin (D,E,F) in the rat sciatic nerve. Two (A,D) or 3 (B,E) wk after radioactive labeling of the spinal cord, cold-soluble and insoluble proteins in 6-mm consecutive segments of the sciatic nerve were separated by SDS-PAGE as described in Methods and shown in Fig. 2C,D. Bands corresponding to tubulin, actin, and NF68 were cut out from stained gels, and radioactivity in each gel piece was measured after extraction with Soluene 350. Distribution of radioactivity associated with soluble (– – –) or insoluble (—) tubulin or actin is plotted as a percentage of total tubulin- or actin-associated radioactivity in the entire length of the nerve analyzed, which were (A) 7800, (B) 17,000, (D) 3200, and (E) 7000, cpm respectively. Radioactivity associated with NF68 (shaded area in A and B) is expressed as a percentage of total tubulin radioactivity for comparison. Representative profiles obtained from a pair of nerves from one animal at each time-point are shown. In (C) and (F), ratio of radioactivity in soluble-to-insoluble tubulin (C) or actin (F) in each segment is shown at 2 wk (– – –) and 3 wk (—) after labeling.

In experiment II, nerves were injured 1 wk after radioactive labeling, so that the response of the cytoskeletal proteins already in transit in the nerve at the time of injury could be studied. In both cases, cytoskeletal proteins were allowed to be transported for 2 wk after radioactive labeling. For each type of experiment, representative transport profiles obtained from a pair of nerves from one of the three animals are shown in Figs. 4 and 5. The results from the other two animals were in good agreement with the one shown, except for slight differences in distribution owing to differences in the location of the injury site, which was 70–80 mm from the spinal cord.

When transport profiles of cold-soluble and cold-insoluble tubulin in the regenerating nerve (Fig. 4A) are compared with those of the control nerve (Fig. 4B) in experiment I, the regenerating nerve was shown to contain a large amount of faster migrating component (54–90 mm from the spinal cord) which was not present in the control nerve at 2 wk postlabeling. The soluble-to-insoluble tubulin ratio of 2.9 for this component was comparable to that of SCb in the control

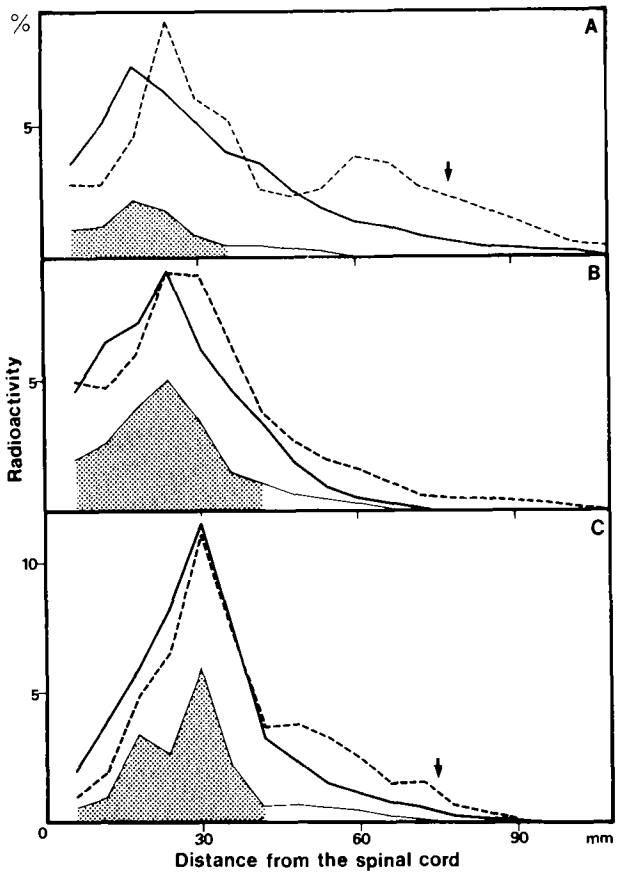

Fig. 4. Comparison of the transport profiles of soluble and insolube tubulin in control nerve (B) and nerves regenerating under conditions defined in experiment I (A) or II (C), 2 wk after radioactive labeling of the spinal cord. (A) Regenerating nerve injured 1 wk prior to labeling, (B) control nerve, (C) regenerating nerve injured 1 wk after labeling. Radioactivity associated with cold-soluble (– – –) or insoluble (—) tubulin was measured as described in Methods and in the legend to Fig. 3, and plotted as a percentage of total tubulin-associated radioactivity in the entire length of the nerve, which was (A) 5000, (B) 7800, and (C) 4100 cpm, respectively. Radioactivity associated with NF68 (hatched) is expressed also as a percentage of total tubulin radioactivity for comparison. Site of injury is indicated with an arrow. For each type of experiment, representative profiles obtained from a pair of nerves from one animal are shown.

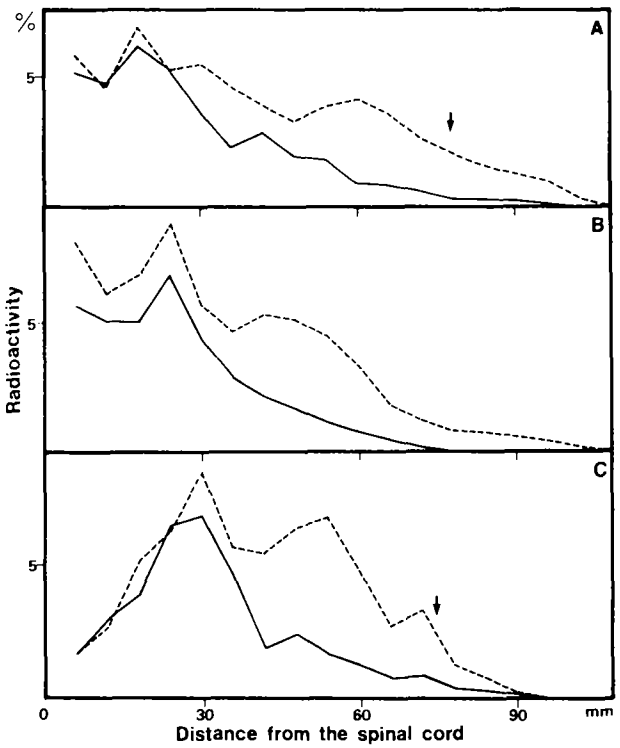

Fig. 5. Comparison of the transport profiles of soluble and insoluble actin in control nerve (B) and nerves regenerating under conditions defined in experiment I (A) or II (C), 2 wk after radioactive labeling of the spinal cord. (A) Regenerating nerve injured 1 wk prior to labeling; (B) control nerve; (C) regenerating nerve injured 1 wk after labeling. Radioactivity associated with cold-soluble (– – –) or insoluble (—) actin was measured and plotted as a percentage of total actin-associated radioactivity in the entire length of the nerve analyzed, which was (A) 2200, (B) 3200, and (C) 1500 cpm, respectively. Site of injury is indicated with an arrow. For each type of experiment, representative profiles obtained from a pair of nerves from one animal are shown.

nerve, indicating an increase in the proportion of SCb as well as the preferential acceleration of this component. The average rate of 4.5 mm/d corresponds to 50% increase in the rate of SCb. The rate of the slower main component was not increased compared with that of SCa in the control nerve. A slight retardation was consistently observed in the transport of the peak of insoluble tubulin and the neurofilament proteins. There was, however, more insoluble tubulin located ahead of SCa in the regenerating nerve, 19% of insoluble tubulin was within 48–90 mm from the spinal cord in the regenerating nerve, whereas only 9% was in this region in the control nerve. The amount of labeled NF68 was decreased in comparison with tubulin.

In experiment II, nerves were injured 1 wk after radioactive labeling when labeled cytoskeletal proteins had advanced well into the axons to observe regeneration-related changes in these preexisting proteins. The front of radioactivity was at 30 mm from the spinal cord at this time interval, whereas the injury site was at 70–80 mm. Thus, the labeled proteins were not directly affected by the injury. As shown in Fig. 4C, a smaller, but significant peak enriched in cold-soluble tubulin was also detected ahead of the main wave (Fig. 4C). Location of the faster component corresponds to that of SCb if it is estimated to be accelerated by 50% under regenerating conditions for 1 wk.

Axonal transport of actin was similarly affected during regeneration as shown in Fig. 5. In experiment I, the faster component was shifted more distally, indicating the preferential acceleration. In experiment II, an increase in the proportion of cold-soluble actin in the faster component was observed. Such coordinate changes observed in axonal transport of both tubulin and actin indicate that the organization of proteins carried in SCb is primarily affected during regeneration.

Discussion

From the biochemical analysis of tubulin and actin transported in the peripheral nerve, we have shown here and in the previous reports (Tashiro and Komiya, 1987,1989) that large proportions of tubulin and actin in the axon exist in the insoluble form, which is resistant to various depolymerizing conditions. Transport profiles of cold-soluble and cold-insoluble tubulin and actin show that they are distributed differentially between the two subcomponents of slow axonal transport, SCa and SCb, resulting in higher solubility of the faster component SCb. The shift of tubulin and actin from SCa to SCb together with the selective acceleration of SCb observed during regeneration further suggests that the cold soluble form is more directly involved in transport than the insoluble form. Such cold-soluble tubulin and actin must derive from dynamic polymers and a pool of nonpolymerized subunits that are in equilibrium.

The biochemically distinguished cold-insoluble tubulin may correspond to short fragments of stable microtubules observed morphologically in the mature axon after treatments to destabilize microtubules (Heidemann et al., 1984; Donoso, 1986; Sahenk and Brady, 1987; Baas and Black, 1990). These stable microtubules have been shown to be enriched in α-tubulin posttranslationally modified by acetylation or detyrosination (Cambray-Deakin and Burgoyne, 1987; Baas and Black, 1990). The dynamic properties of such stable microtubules are largely unknown, since most of the studies on microtubules have been carried out using brain microtubules obtained from the soluble pool through cycles of assembly and disassembly that completely lack the stable subpopulation. It has been shown recently that cycled brain microtubules are depleted of acetylated α-tubulin (Kim, 1991). The stable microtubule fragments may play an important role in maintaining axonal structure by serving as seeds for local repolymerization, since microtubule organizing centers are not found in the axon.

In regenerating nerve, large changes are known to take place in cytoskeletal protein synthesis at the cell body (for review, *see* Bisby and Tetzlaff, 1992) that influence the composition as well as the rate of slow axonal transport (Grafstein and Murray, 1969; Hoffman and Lasek, 1980; Hoffman et al., 1985b; Oblinger and Lasek, 1988). A reduction in neurofilament protein synthesis and an increase in tubulin and actin synthesis are observed (Hoffman et al., 1987; Hoffman and Cleveland, 1988; Oblinger and Lasek, 1988; Tetzlaff et al., 1988,1991; Miller et al., 1989). Among the various tubulin isotypes expressed in the neuron, Tα1 mRNA for α–tubulin (Mlller et al., 1989) and type II mRNA for β-tubulin (Hoffman et al., 1987; Hoffman and Cleveland, 1988), both of which are expressed at high levels during development (Hoffman and Cleveland, 1988; Miller et al., 1988), are specifically increased in the axotomized neuron. It can be concluded

from the present study that such an altered set of cytoskeletal proteins synthesized in response to nerve injury has increased solubility and is associated more with SCb.

Peripheral neurons respond to axotomy by active regeneration following a lag period of 1–4 d (Forman and Berenberg, 1978; Forman et al., 1979; Komiya, 1981a; McQuarrie and Lasek, 1989). Since the rate of slow axonal transport is not fast enough for the new set of proteins synthesized in response to injury to reach the injury site within the lag period, local reorganization of the preexisting cytoskeleton must be responsible for the formation of the growth cone and the initial sprouting (Komiya, 1981b; McQuarrie and Lasek, 1989; Tetzlaff and Bisby, 1989). It has been shown in vitro that a severed distal fragment of a neurite is able to form a new growth cone and move for hours (Shaw and Bray, 1977). The results of experiment II further indicate that such a reorganization of the preexisting cytoskeleton takes place not only at the injury site, but possibly throughout the length of the axon, since the cytoskeletal proteins located more than 40 mm away from the injury site were affected. It also seems impossible to avoid discontinuity of the axon because of accumulation or depletion of cytoskeletal components if the rate of transport changed only locally.

At present, there are controversial views concerning the actual transport form of tubulin, whether it translocates as polymerized microtubules (Black and Lasek, 1980; Keith, 1987; Reinsch et al., 1991) or as depolymerized subunits that are then exchanged with the stationary polymers (Komiya and Tashiro, 1988; Okabe and Hirokawa, 1988,1990; Ochs et al., 1989; Tashiro and Komiya, 1989; Lim et al., 1990). It is clear, however, that destabilization of cytoskeletal polymers (increase in their solubility), such as seen during regeneration, results in acceleration of transport, whereas the increase in polymer stabilization observed during maturation and aging leads to reduction in the rate of transport (Komiya, 1981b; Hoffman et al., 1985a; McQuarrie et al., 1989; Watson et al., 1990; Tashiro and Komiya, 1991b). Thus, the key question will be to find out the molecular mechanisms that control the stability of cytoskeletal polymers, especially of microtubules in the axon. Posttranslational modifications of tubulin, such as acetylation, detyrosination, and polyglutamylation, which are most prominent in the nervous system (Gozes and Sweadner, 1981; Gard and Kirschner, 1985; Black and Keyser, 1987; Baas and Black, 1990; Eddé et al., 1990; Alexander et al., 1991), may be one of the mechanisms controlling the stability of microtubules directly, or indirectly by altering the extent of interactions with neurofilaments or other regulatory proteins.

Acknowledgment

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