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Cytochemical evidence for redistribution of membrane pump calcium-ATPase and ecto-Ca-ATPase activity, and calcium influx in myelinated nerve fibres of the optic nerve after stretch injury

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Summary

There has been controversy for some time as to whether a posttraumatic influx of calcium ions occurs in stretch/non-disruptively injured axons within the central nervous system in both human diffuse axonal injury and a variety of models of such injury. We have used the oxalate/pyroantimonate technique to provide cytochemical evidence in support of such an ionic influx after focal axonal injury to normoxic guinea pig optic nerve axons, a model for human diffuse axonal injury. We present evidence for morphological changes within 15 min of injury where aggregates of pyroantimonate precipitate occur in nodal blebs at nodes of Ranvier, in focal swellings within axonal mitochondria, and at localized sites of separation of myelin lamellae. In parallel with these studies, we have used cytochemical techniques for localization of membrane pump Ca^{2+}-ATPase and ecto-Ca-ATPase activity. There is loss of labelling for membrane pump Ca^{2+}-ATPase activity on the nodal axolemma, together with loss of ecto-Ca-ATPase from the external aspect of the myelin sheath at sites of focal separation of myelin lamellae. Disruption of myelin lamellae and loss of ecto-Ca-ATPase activity becomes widespread between 1 and 4 h after injury. This is correlated with both infolding and retraction of the axolemma from the internal aspect of the myelin sheath to form periaxonal spaces which are characterized by aggregates of pyroantimonate precipitate, and the development of myelin intrusions into invaginations of the axolemma such that the regular profile of the axon is lost. There is novel labelling of membrane pump Ca^{2+}-ATPase on the cytoplasmic aspect of the internodal axolemma between 1 and 4 h after injury. There is loss of an organized axonal cytoskeleton in a proportion of nerve fibres by 4–6 h after injury. We suggest that these changes demonstrate a progressive pathology linked to calcium ion influx after stretch (non-disruptive) axonal injury to optic nerve myelinated fibres. We posit that calcium influx, linked to or correlated with changes in Ca^{2+}-ATPase activities, results in dissolution of the axonal cytoskeleton and axotomy between 4 and 6 h after the initial insult to axons.

Introduction

Cytochemical and morphological evidence for entry of Ca^{2+} into axons after crush injury to the sciatic nerve (Mata *et al.*, 1986), after weight drop injury to the surgically exposed spinal cord (Ballentine, 1978; Happell *et al.*, 1981) and electrophysiological evidence in either anoxic/ischaemic injury to the optic nerve (Stys *et al.*, 1992) or contused spinal cord (Stokes *et al.*, 1985) provides support for the hypothesis that calcium influx into myelinated nerve fibres is a key event in the development of the subsequent pathology in injured, mammalian nerve fibres. However, the hypothesis that calcium influx also occurs in diffuse axonal injury (DAI) where the majority of axons are, probably, not sheared at the time of injury, but rather undergo 'secondary axotomy' (Maxwell *et al.*, 1993), remains speculative (Povlishock, 1992). A recent paper (Pettus *et al.*, 1994) has provided the first morphological evidence that the axolemma becomes permeable to relatively large molecules, for example tracers such as horseradish peroxidase, after traumatic brain injury (TBI). It is also clear that there are major differences between grey and white matter in the CNS regarding

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possible mechanisms for Ca^{2+} influx, in particular after irreversible anoxic/ischaemic injury (Choi, 1985; Krieglstein *et al.*, 1989; Weiss *et al.*, 1990; Stys *et al.*, 1992), and that the mode(s) of entry of Ca^{2+} into cells has not been fully characterized.

Two transport mechanisms have been identified for the extrusion of Ca^{2+} ions from myelinated nerve fibres of mammals, the Na^+/Ca^{2+} exchanger system which is dependent upon the maintenance of the Na^+ gradient and regulated by intracellular Ca^{2+}, and ATP-dependent Ca^{2+} pump(s) (Blaustein, 1988). The molecular identity and structure of the Na^+/Ca^{2+} exchanger molecule has still not been determined (Lagnado & McNaughton, 1990) but there is a concensus that it is a single protein of about 225 kDa (Hale *et al.*, 1988). Electrophysiological and ultrastructural evidence suggests that the exchanger provides a mechanism for Ca^{2+} influx under conditions of depolarization and/or anoxia/ischaemia (Blaustein, 1988; Stys *et al.*, 1992; Waxman *et al.*, 1994). But, it has been posited (Stys *et al.*, 1992), that under normoxia the supply of ATP is not limited and that Ca^{2+}-ATPase would be capable of extruding most of the excess Ca^{2+} admitted through reverse Na^+/Ca^{2+} exchange (Stys *et al.*, 1992).

There are two recognized membrane pump Ca^{2+}-ATPases. One is located in the plasma membrane with its ATP binding site located on the axoplasmic aspect of the membrane (Kondo *et al.*, 1988; Mata & Fink, 1989; Gioglio *et al.*, 1991; Pappas & Kriho, 1991). It is calmodulin-sensitive and due to its high affinity for calcium it can reduce the cytoplasmic concentration of free calcium ions to extremely low levels (for review see Rega & Garrahan, 1986). The second is calmodulin-insensitive and serves to sequester Ca^{2+} into the smooth endoplasmic reticulum/calciosome system (Carafoli, 1987). Under resting conditions, it has been posited, much of the net Ca^{2+} extrusion from the axon is probably achieved by the ATP dependent axolemma pump (Blaustein, 1988; Stys *et al.*, 1992). In addition, however, recent evidence has demonstrated (Ca^{2+}–Mg^{2+})-ATPase activity in association with cell adhesion molecules, for example neural cell adhesion molecule (NCAM) (Dzhandzhugazyan & Bock, 1993). These have been termed ecto-Ca-ATPases and may be distinguished from membrane pump ATPases where appropriate cytochemical techniques are used (Kortje *et al.*, 1990; Barry, 1992). The function of ecto-Ca-ATPases is not presently understood (Barry, 1992) but the role of Ca^{2+} in the integrity of the myelin sheath (Inouye & Kirschner, 1988; Ropte *et al.*, 1990) for example, make them worthy of consideration in pathological changes in injured, myelinated nerve fibres.

Of course, under conditions of anoxia Ca^{2+}-ATPase activity, both the membrane pump and the ecto-Ca-ATPase, will be reduced due to a lack of availability of ATP. However, no evidence to substantiate earlier suggestions that ischaemic (anoxic) insults may provide a mechanism for axonal damage (Jellinger & Seitelberger, 1970) has been obtained from experimental models of diffuse/traumatic head injury (Povlishock, 1992). Indeed, detailed ultrastructural analyses of the related parenchyma of the brain or optic nerve, and the microvasculature, have failed to conclusively show that traumatically induced ischaemia is a factor in the genesis of axonal abnormalities (Povlishock, 1986; 1992; Maxwell *et al.*, 1991c, 1992). As already mentioned above, it has been posited that under normoxia the supply of ATP is not limited and that Ca^{2+}-ATPase would be capable of extruding most of the excess Ca^{2+} admitted through reverse Na^+/Ca^{2+} exchange (Stys *et al.*, 1992). Thus, we argue, that although there is strong evidence for reversed activity of the Na^+/Ca^{2+} exchanger in anoxia/ischaemia (Stys *et al.*, 1992; Waxman *et al.*, 1994), evidence for such has not been provided under conditions of normoxia in models of DAI or TBI. We therefore decided to study alterations in membrane pump Ca^{2+}-ATPase and ecto-Ca-ATPase activity after CNS injury in our stretch injury model in the guinea-pig optic nerve. We have utilized the pyroantimonate technique (Borgers *et al.*, 1977) to cytochemically demonstrate the localization of calcium, and modified the lead citrate technique for the cytochemical localization of Ca^{2+}-ATPase activity (Ando *et al.*, 1981) to investigate changes in membrane pump and ecto-Ca^{2+}-ATPase distribution in axons and the myelin sheaths of myelinated fibres after stretch/non-disruptive axonal injury.

Materials and methods

Under intramuscular ketamine (50 mg kg^{-1}) and xylazine (3 mg kg^{-1}) anaesthesia, the right optic nerve of 60 adult guinea-pigs (range 700–850 g) was stretched, in a controlled manner, to provide reproducible and measurable amounts of elongation or tensile strain (Gennarelli *et al.*, 1989). Those animals that were allowed to survive for a period of less than 4 h after injury were maintained under light, Halothane (1% in oxygen) anaesthesia until they were killed, in order to minimise post-operative discomfort. Animals surviving for periods in excess of 4 h resumed normal feeding and motor behaviour, and were examined at regular intervals after injury. Six animals were used as controls in that stretch injury was not applied to the optic nerve before they were fixed for cytochemical visualisation of reaction product. At selected intervals after injury (15 min, 1, 2, 4, 24, 48 h and 7 days), animals were terminally anaesthetized with IP Sagatal. After all respiratory movement had ceased, a thoracotomy was followed by perfusion through the left ventricle with fixatives.

Thirty-six animals were fixed to allow cytochemical demonstration of Ca^{2+}-ATPase activity by use of 0.1 M cacodylate buffer, adjusted to pH 7.3 with potassium hydroxide, at 37° C for 1 min. This was followed by 4% paraformaldehyde in 0.1 M cacodylate buffer with 6% sucrose

added (1450–1520 mosmol) (pH 7.3) at 4° C for approximately 30 min. After perfusion, optic nerves were dissected out and each divided into three equal segments which were subsequently processed separately. The segments were immersion fixed in the same fixative for 5 h at 4° C. Segments were embedded in Agar and cut longitudinally into 50 μm sections on a Vibratome. The sections were incubated for 30 min at room temperature in a medium consisting of either 70 mM Tris-maleate (pH 7.4) or 250 mM glycine buffer (pH 9.0) and containing 3 mM ATP, between 0.5 and 12 mM $CaCl_2$ ($n = 6$ at 12 mM, $n = 6$ at 5 mM, $n = 12$ at 1 mM, and $n = 12$ at 0.5 mM $CaCl_2$), and either 0.5 mM bromotetramisole oxalate or 10 mM levamisole (modified after Ando *et al.*, 1981; Maxwell *et al.*, 1991a). After incubation the sections were washed in 0.1 M cacodylate buffer containing sucrose (pH 7.3) at 4° C for 2 h and then postfixed in cacodylate buffered 1% osmium tetroxide with 3% sucrose for 20 min at room temperature. Sections were then routinely dehydrated through a graded series of ethanols, propylene oxide and embedded in Araldite. Several control procedures were used on a selection of sections being processed at the same time. Either $CaCl_2$ was deleted and 10 mM EDTA added to the incubation medium, or ATP was omitted from the incubation medium, or sections were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h before washing and exposure to the enzyme reaction. Material was obtained from three control animals on which an operation had not been performed, and three animals at 15 min, 1, 2, 4, and 24 h after injury respectively.

Twenty-four animals (three controls and 21 stretch injured) were fixed for the ultrastructural demonstration of the mobile pool of Ca^{2+} (Borgers *et al.*, 1977) but with slight modification in that 0.5% paraformaldehyde and 1.9% sucrose was added to the fixative (Maxwell *et al.*, 1991a). Three animals at each time point (15 min, 1, 2, 4, 24, 48 h and 7 days, and three controls) were terminally anaesthetized with IP barbiturate and, after thoracotomy, perfused through the left ventricle with 90 mM potassium oxalate in 1.9% sucrose, adjusted to pH 7.4 with potassium hydroxide, at 37° C for 2 min, followed by 3% glutaraldehyde, 0.5% paraformaldehyde, 90 mM potassium oxalate, 1.9% sucrose (750–850 mosmol) adjusted to pH 7.4 with potassium hydroxide for 1 h. The first 500 ml of the fixative was warmed to 37° C and perfused rapidly in contrast to the remaining 2000 ml which was cooled to approximately 4° C and perfused more slowly. After perfusion, both optic nerves (the left nerve to be used as an internal control) were dissected out and each divided into three equal segments which were subsequently processed separately. The segments were placed in the same fixative at 4° C for 2 h, briefly rinsed in 90 mM potassium oxalate in 1.9% sucrose (pH 7.4) and postfixed in 1% osmium tetroxide and 2% potassium pyroantimonate for 2 h at room temperature. Unreacted pyroantimonate was washed out with distilled water adjusted to pH 10 with potassium hydroxide for 15 min. The segments were then routinely dehydrated through a series of 50%, 70%, 90% and three times 100% ethanols for 40 min before being cleared in two 20 min changes of propylene oxide. Finally, the segments were placed successively in 1:1 propylene oxide/Araldite, 1:2 propylene oxide/Araldite, two 4 h changes of pure Araldite before embedding in pure Araldite polymerized at 60° C. Three control techniques were utilized to demonstrate the specificity of the pyroantimonate technique. In the first control, 2% potassium pyroantimonate was deleted from the postfixation medium. In the second, thin sections supported on copper grids were washed in 10 mM EDTA at 60° C for 1 h. In the third control, electron probe microanalysis (EPXMA) was performed on thin sections of gold interference colour cut wet using a diamond knife and a Reichart-Jung E Ultracut ultramicrotome. The sections were taken from material that had been incubated in medium either containing or lacking potassium pyroantimonate, and were mounted on titanium single hole grids with a Formvar support film. Sections were unstained to minimise background and to avoid extraneous characteristic peaks. Analysis was carried out in a modified Jeol 100C transmission electron microscope (Nicholson *et al.*, 1982) equipped with a 30 mm^2 Kevex energy dispersive detector and Link systems 290 X-ray analysis system using an accelerating voltage of 80 keV, a magnification of ×10 000, a collection time of 200 s, and a specimen tilt 30° from the horizontal. The zero stroke peak and TiKα line were used to calibrate the analyser for gain and zero shifts.

For routine examination of thin sections the sections were examined, unstained, in either a Jeol 100s or a Philips 301 transmission electron microscope.

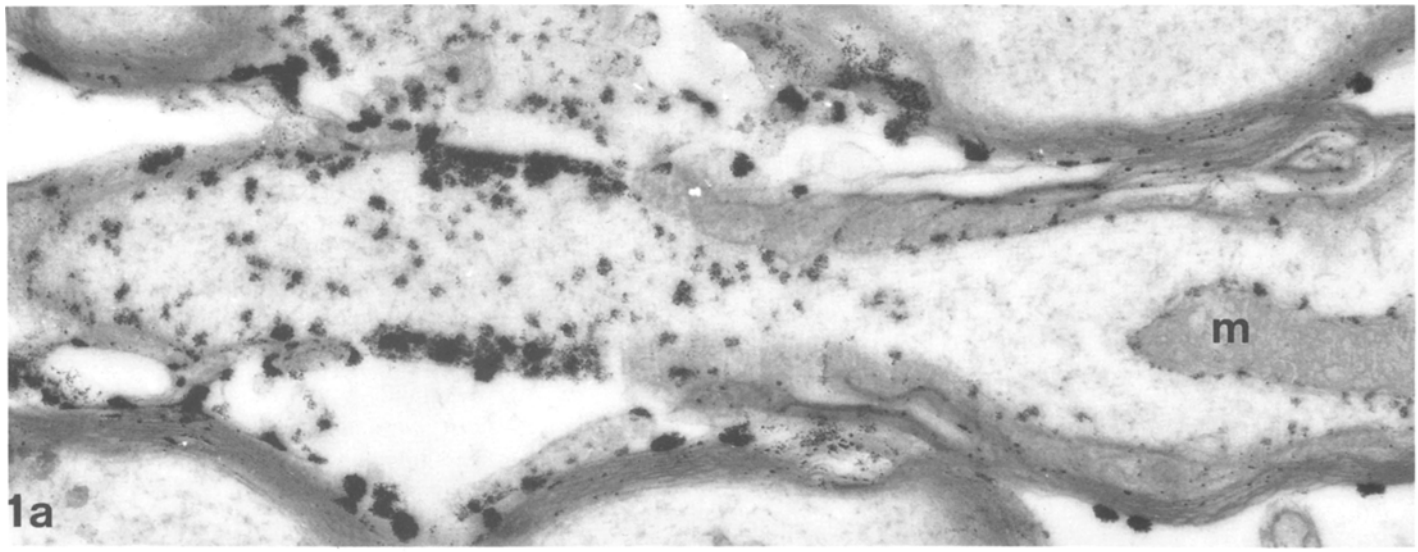
Results

Control animals

After incubation of sections in a medium containing 1 or 0.5 mM $CaCl_2$ reaction product indicating Ca^{2+}-ATPase activity was always localized on cytoplasmic/axoplasmic membrane surfaces. No reaction product occurred on the axolemma except at the node of Ranvier (Fig. 1a). At this site, precipitate was located on the axoplasmic surface of the nodal axolemma such that the precipitate had an irregular profile on the axoplasmic side, but was always sharply delineated on the extra-axonal aspect. Within the axoplasm, reaction product was found on the external and cristal membranes of the mitochondria and membranous profiles of the smooth endoplasmic reticulum (SER).

Reaction product occurred at three sites in the myelin sheath in sections incubated in media containing 12 mM $CaCl_2$, that is, conditions which identify ecto-Ca-ATPase activity. A fairly uniform deposit occurred on the outer surface of the compact myelin, occasional deposits occurred at a minority of areas of separation of the myelin membranes, possibly Schmidt-Lanterman incisures (SLI) (Mata *et al.*, 1986; Maxwell *et al.*, 1991a), and a highly ordered reaction product on the membranes of the paranodal loops of the myelin sheath (Fig. 1b,c).

Calcium pyroantimonate precipitate occurred diffusely throughout the axoplasm of myelinated fibres. In the internodal region the precipitate appeared to be randomly distributed (Fig. 2a).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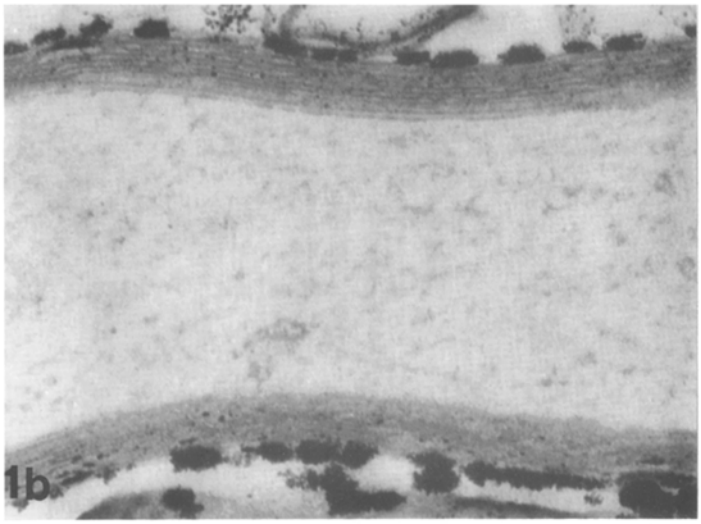
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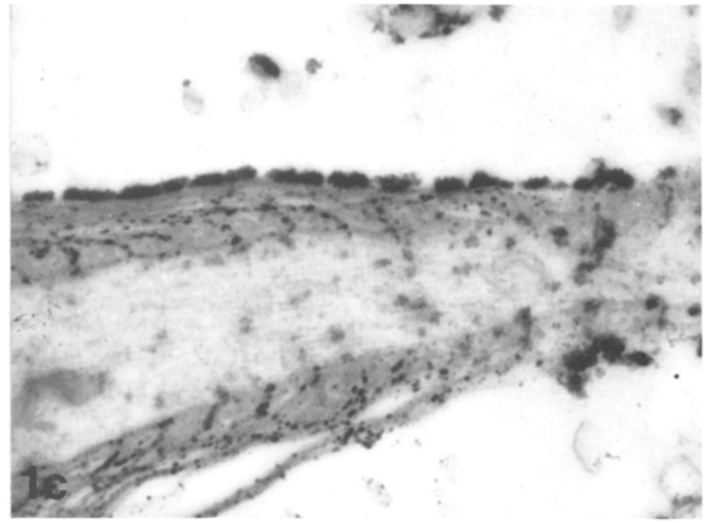
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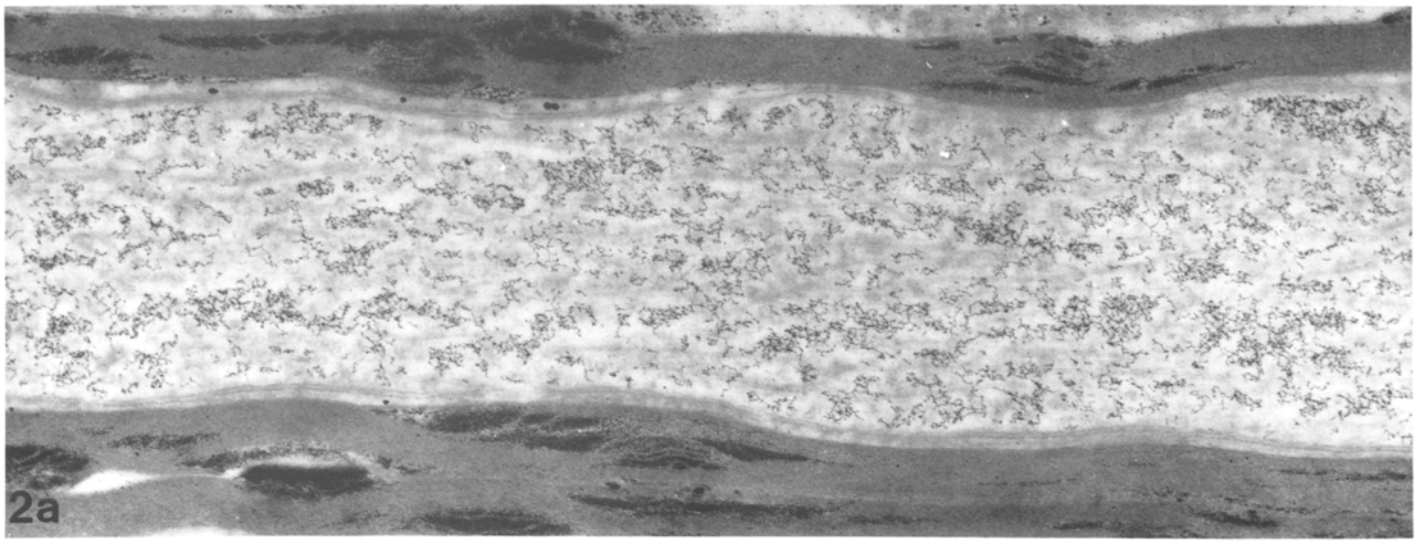
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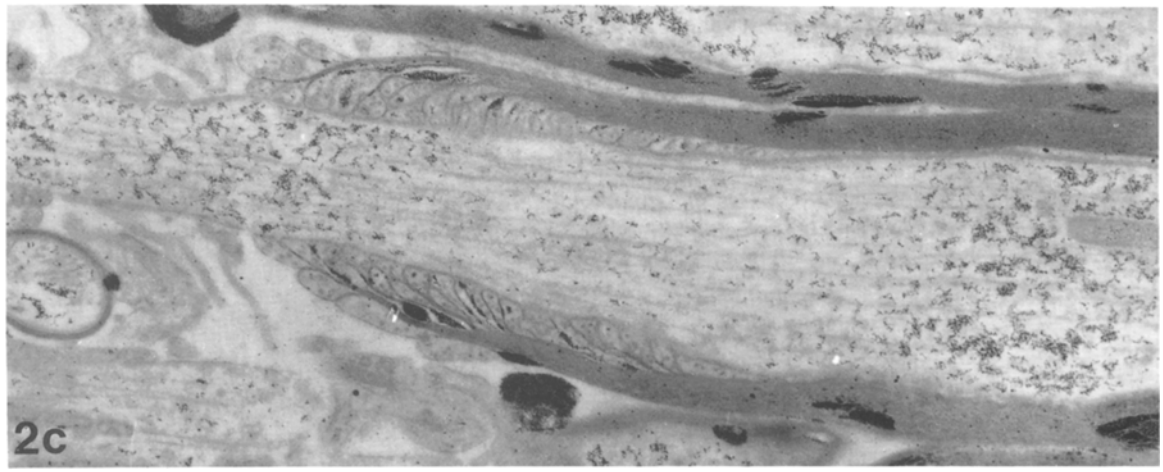
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Precipitate usually occurred over mitochondria but the density of the precipitate was relatively low (*vide infra*) and mitochondrial morphology was normal (Fig. 2b). Precipitate also occurred in association with some membranous profiles of endoplasmic reticulum. There was no discrete association with the axolemma. The region of the node of Ranvier was characterized by a gradient of reduced precipitate (Maxwell *et al.*, 1991a) with decreased content in the axoplasm underlying the paranodal glial loops (Fig. 2c) while present at the node and the internode. In the myelin, Ca^{2+} precipitate occurred in the paranodal glial loops adjacent to the node of Ranvier, and where the lamellae of the myelin sheath were dissociated, for example at Schmit-Lanterman incisures.

Lesioned animals

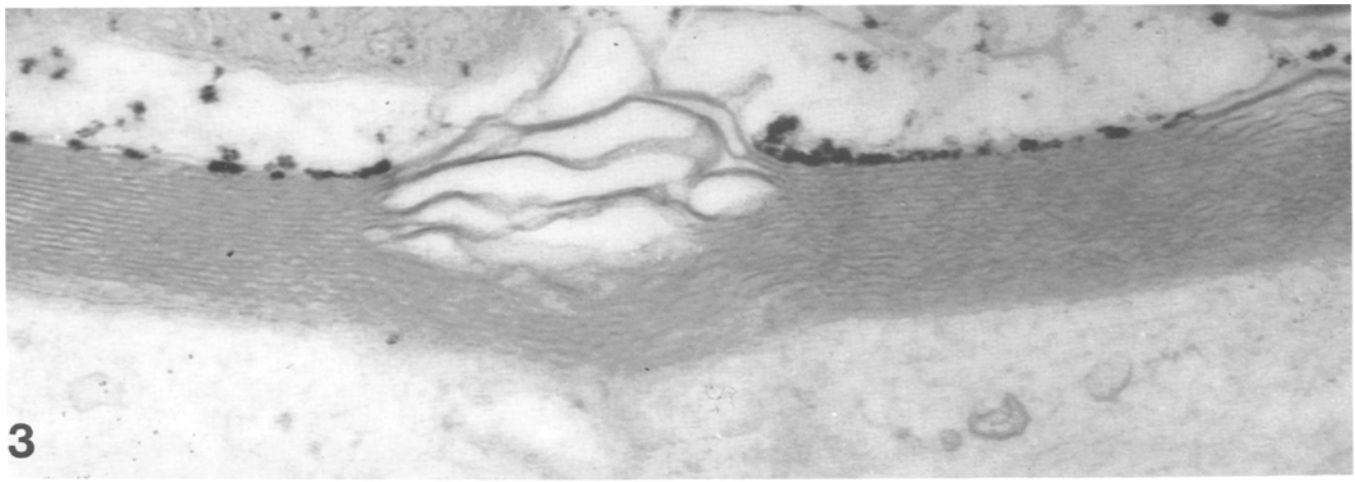
A regular finding common to material processed either for the localisation of Ca^{2+}-ATPase activity or calcium pyroantimonate was a localized loosening of the internodal myelin lamellae 15 min after stretch injury. This loosening was associated with a decrease or even complete loss of labelling for ecto-Ca-ATPase activity, in particular where the incubation medium contained 5–12 mM $CaCl_2$ (Fig. 3), which otherwise featured prominently on the external surface of the compact myelin in the internodal portion of the axon. Loosening (or dissociation) of myelin lamellae was not observed in control material. At the sites of myelin dissociation, the myelin intruded into the regular profile of the axolemma to result in the occurrence of irregular, membranous profiles. There was increased content of pyroantimonate precipitate at these sites (Fig. 4). Intra-axonally the most notable change 15 min after stretch injury was the accumulation of aggregates of pyroantimonate precipitate on the deep aspect of the myelin sheath and a partial loss of a recognisable axonal cytoskeleton (Fig. 4). Frequently, pryoantimonate precipitate occurred in the matrix of mitochondria which demonstrated a focal increase in transverse diameter such that the mitochondria were often characterized by circumscribed, bulbous swellings somewhere along their length (Fig. 5).

The occurrence of so-called 'nodal blebs' has been previously documented 15 min after stretch injury (Maxwell *et al.*, 1991b). In nodes demonstrating nodal blebs aggregates of pyroantimonate precipitate occurred within the axoplasm (Fig. 6), in particular within the bleb. However, we did not obtain convincing evidence for any reduction in density of precipitate denoting membrane pump Ca^{2+}-ATPase activity 15 min after injury.

One, 2 and 4 h after stretch injury evidence for reduced nodal/paranodal labelling for Ca^{2+}-ATPase activity was obtained when the incubation medium contained 0.5–1 mM $CaCl_2$. In some examples of longitudinal sections of nodes of Ranvier there was a complete absence of labelling for Ca^{2+}-ATPase activity on the axoplasmic aspect on the nodal axolemma (Fig. 7), while in adjacent nodes within the same sections deposits of reaction product were associated with the nodal axolemma. These findings provide support for the concept that individual nerve fibres within larger groups of fibres undergo a spectrum/progression of pathological changes after traumatic brain injury such that heterogeneous and complex pathological processes may be involved in reactive axonal change occurring both in the internodal regions (Pettus *et al.*, 1994) and nodes of Ranvier after stretch/non-disruptive axonal injury (Maxwell *et al.*, 1991b; Maxwell, 1995). The impression of a spectrum of pathological changes was more notable when optic nerves were examined in transverse section. One hour after stretch injury there was a more widespread loosening of myelin lamellae than was noted at 15 min such that spaces occurred between the axolemma and the inner aspect of the myelin sheath. We shall term this space the 'periaxonal space'. In addition, myelin intrusions, as defined by Ochs and Jersild (1990), extended into the periaxonal spaces between the axolemma and the inner aspect of the myelin sheath. Myelin intrusions also extended into invaginations of the axolemma. In material processed for pyroantimonate localization of calcium, extensive deposits of pyroantimonate precipitate were associated with the areas of dissociated/separated myelin lamellae and myelin intrusions (Fig. 8). The axonal cytoskeleton within axons ranged from a structure comparable to that of control axons, through an increased spacing, or reduced density between elements of the cytoskeleton

Fig. 1. (a) Longitudinal section of a node of Ranvier and part of the paranode of a myelinated fibre from a control animal where the section was incubated in 0.5 mM $CaCl_2$. Dense aggregates of reaction product are localized on the axoplasmic side of the nodal axolemma. There is diffuse labelling on intra-axoplasmic organelles. (*m* = mitochondrion). The section was examined unstained ×40 000. (b,c) Longitudinal sections of (b) the internodal region and (c) paranodal regions of myelinated fibres from a control animal. The section was incubated in 12 mM $CaCl_2$ and examined unstained. Reaction product is localized to (b) the external aspect of the myelin sheath and (c) the external aspect of the paranodal myelin and paranodal glial loops. ×33 000.

Fig. 2. Sections taken from control animals and processed for the pyroantimonate technique for calcium. Sections were examined unstained. 2(a) a longitudinal section of an internodal portion of a fibre, 2(b) of a mitochondrion within the internodal axoplasm and 2(c) a node and paranode of a myelinated fibre. Pyroantimonate precipitate occurs (a) diffusely throughout the internodal axoplasm, (b) in only small quantities over the mitochondrion and (c) over the nodal and internodal, but not the paranodal axoplasm. (a) ×39 500; (b) ×24000; (c) ×23 000.



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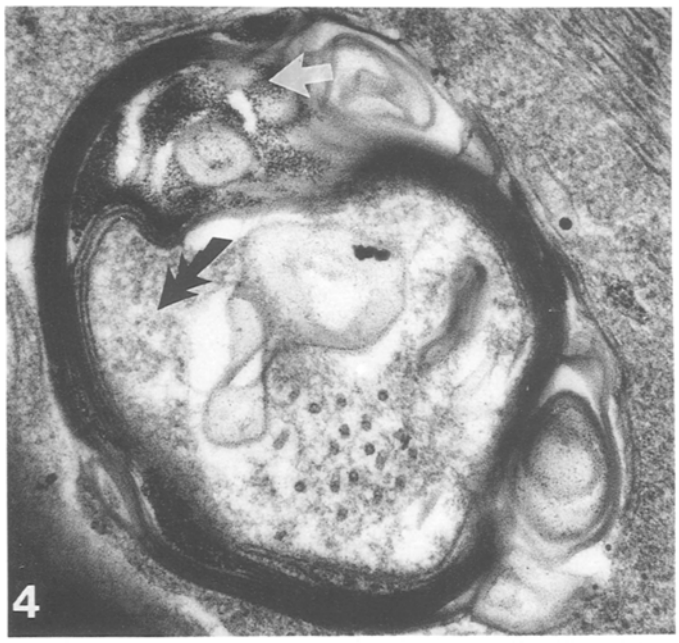

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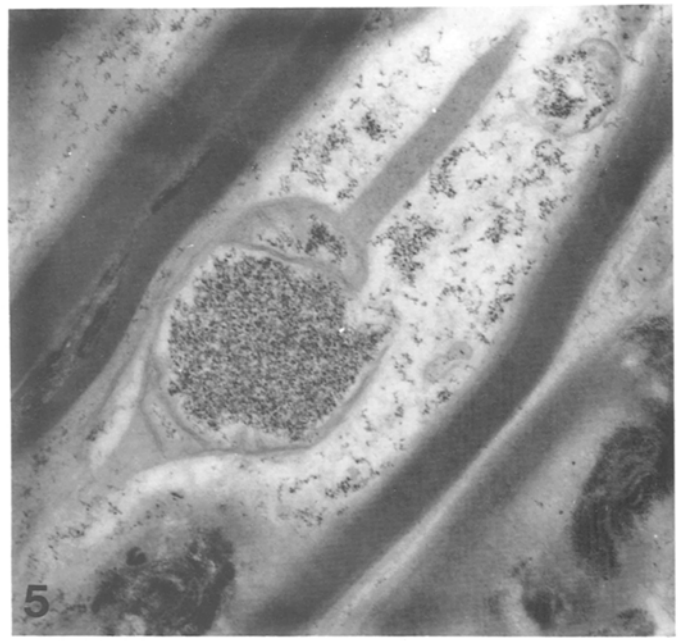

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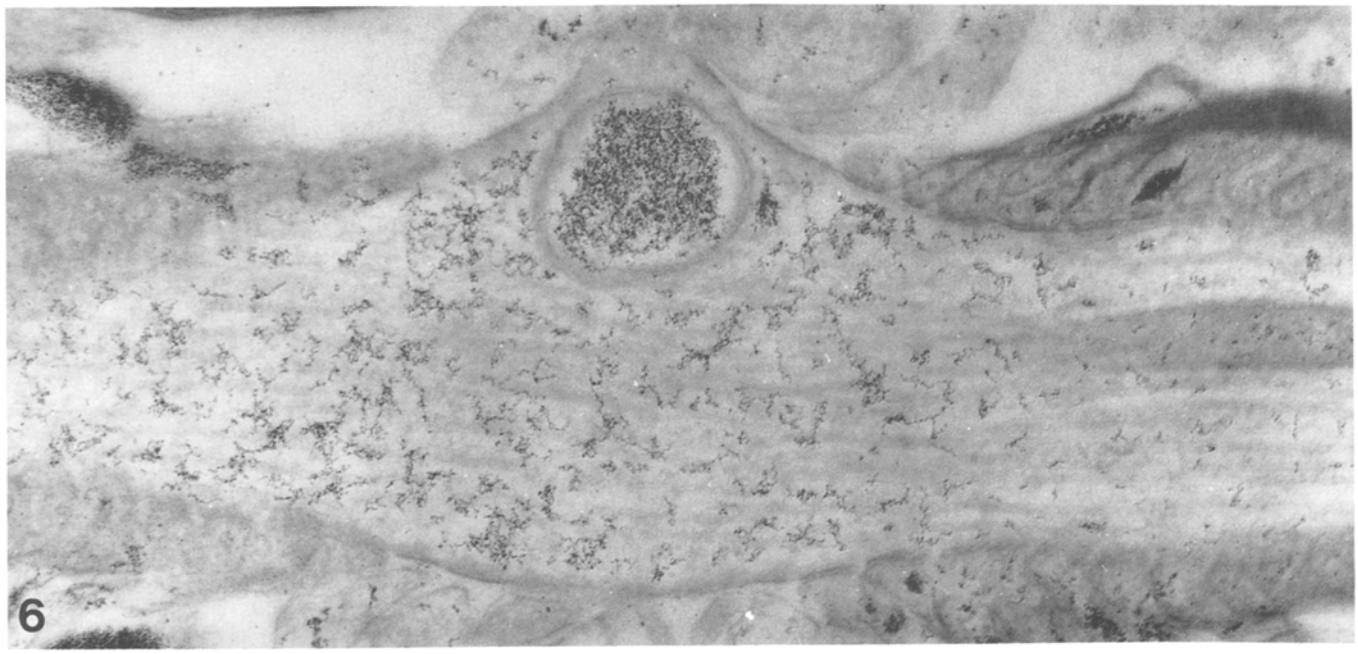

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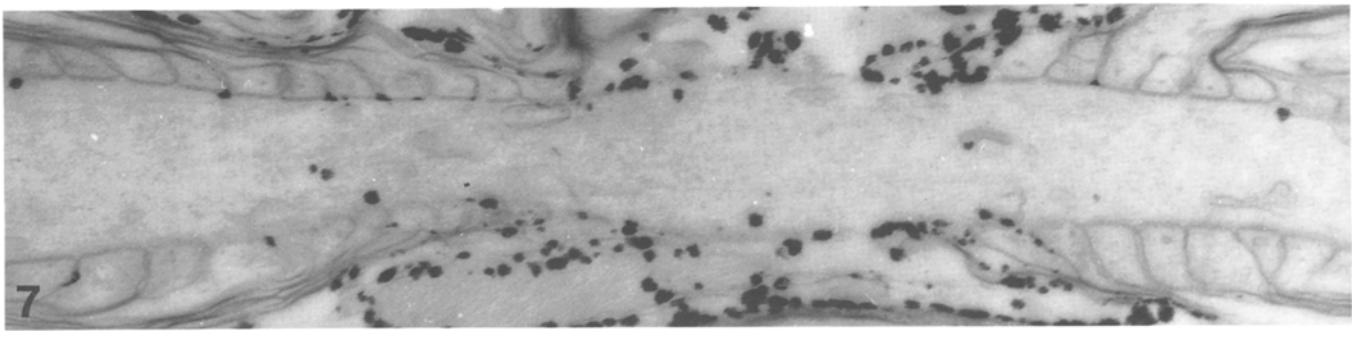

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together with a loss of regular, linear orientation of the neurofilaments and microtubules (Fig. 9), to the occurrence of a flocculent ultrastructure (Fig. 10). In a low number of fibres there was a complete absence of material within the myelin sheath suggestive of total destruction of the axon or axotomy. Axotomy, as reflected by a complete loss of a recognizable axonal cytoskeleton was first noted 4 h after stretch injury and with increasing frequency with a greater survival.

One hour after stretch injury, the most striking finding was the occurrence of Ca^{2+}-ATPase activity on the internodal axolemma. This was manifested as the occurrence of a fine deposit of reaction product on the axoplasmic aspect of the axolemma when incubated with 0.5–1 mM $CaCl_2$ (Fig. 11a). Labelling for membrane pump Ca^{2+}-ATPase activity occurred on the cytoplasmic aspect of the irregularly profiled axolemma, which formed invaginations into the axoplasm (Fig. 11a). Within the related axoplasm occurred aggregates of membranous organelles. Four hours after injury there was heavy labelling on the axoplasmic aspect of the internodal axolemma (Fig. 11b). The separation/dissociation of myelin lamellae was now more widespread. Pyroantimonate precipitate was conspicuous in both areas of myelin dissociation and in the periaxonal space (Fig. 11c).

Examination of transverse sections of axons 4 h after stretch injury revealed areas of discontinuity of the axolemma (Fig. 12a) suggestive of fragmentation of the axolemma. We suggest that since these were present in material routinely fixed for electron microscopy they demonstrate the occurrence of 'holes' within the axolemma rather than being transient openings of the plasmamembrane that have been documented elsewhere (Yu & McNeil, 1992). In addition, some internodes exhibited what appeared to be a focal increase in transverse axonal diameter which was thought not to reflect a plane of section artefact (Fig. 12b) but a vital response to the injury. Such focal enlargements of axonal diameter have been termed 'axonal swellings' by other workers (Povlishock, 1992) and we shall refer to them as such. Pyroantimonate precipitate and aggregates of membranous organelles occurred within their axoplasm (Fig. 12b). The axolemma in these regions often possessed an irregular profile reflecting the occurrence of infoldings or lobulation of the axolemma which has been reported elsewhere (Povlishock, 1992). There was also separation of the axolemma from the internal aspect of the myelin sheath forming periaxonal spaces. These spaces contained deposits of pyroantimonate precipitate (Fig. 12b). There was still a suggestion of a longitudinal, linear organisation of the components of the axonal cytoskeleton in the centre of these axonal swellings. But the concentration of pyroantimonate precipitate tended to be greater towards the periphery of the swellings (Fig. 12b). In some swellings there was an almost complete loss of an organised cytoskeleton. Rather, swellings were characterised by aggregates of membranous organelles, many of which contained concentrations of pyroantimonate precipitate (Fig. 12c).

Twenty-four hours after stretch injury, the most notable, additional structural change was an increase in the longitudinal dimensions of nodes of Ranvier (Fig. 13). The nodal axolemma was not fragmented but formed a continuum. However there was a loss of both subaxolemma density (Fig. 13) and labelling for Ca^{2+}-ATPase activity. The enlarged nodes of Ranvier had an irregular profile. Their axoplasm contained aggregates of membranous organelles, principally mitochondria (Fig. 13) some of which possessed focal, electron lucent enlargements along their length (Fig. 13). Some paranodal glial loops were electron lucent with an increased diameter, others were spatially separated from the axolemma (Fig. 13). Axonal swellings were also numerous 24 h after stretch injury. Their structure and the localization of pyroantimonate precipitate was

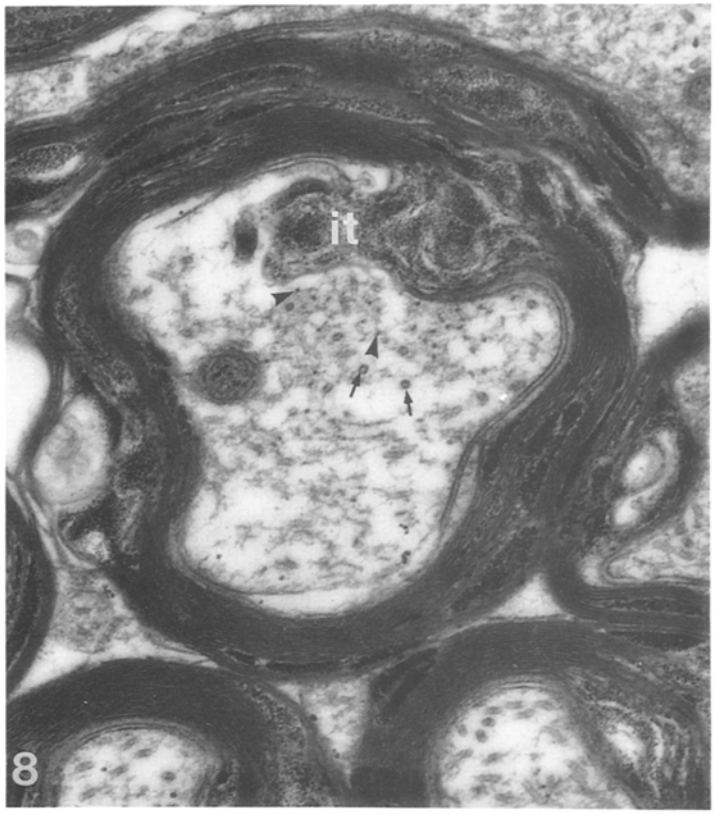
Fig. 3. A longitudinal section of part of the myelin sheath of stretch injured fibre 15 min after injury. The section was incubated in 12 mM $CaCl_2$ and examined unstained. There is focal loosening/separation of myelin lamellae and reaction product is absent in this region. Reaction product is, however, found where the myelin sheath is morphologically intact. ×59 000.

Fig. 4. A transverse section of a myelinated fibre 15 min after stretch injury. The animal was processed for the pyroantimonate technique and the sections examined unstained. The myelin sheath is disrupted and focal aggregates of pyroantimonate precipitate (arrows) occur where the myelin lamellae are separated. Precipitate also occurs in the axoplasm (double arrows), microtubules only occur in a small area of the axoplasm and the axolemma is irregular in profile. ×62 000.

Fig. 5. A longitudinal section of a mitochondrion from a myelinated fibre 15 min after stretch injury. The animal was processed for the pyroantimonate technique and the section examined unstained. The mitochondrion contains an area of increased transverse diameter and within the swollen portion of that mitochondrion is a concentration of pyroantimonate precipitate. ×36 500.

Fig. 6. A longitudinal section of a node of Ranvier with a nodal bleb from a myelinated fibre 15 min after stretch injury. The animal was processed for the pyroantimonate technique and the section examined unstained. There is a concentration of pyroantimonate precipitate within a vesicular profile, possibly a mitochondrion, within the axoplasm of the bleb. ×50 500.

Fig. 7. A longitudinal section of a node of Ranvier from an optic nerve 1 h after stretch injury. The section was incubated in 1 mM $CaCl_2$ and examined unstained. There is patchy localisation of reaction product on glial membranes. But there is no labelling on the axoplasmic aspect of the nodal axolemma (cf with Fig. 1a). ×32 500.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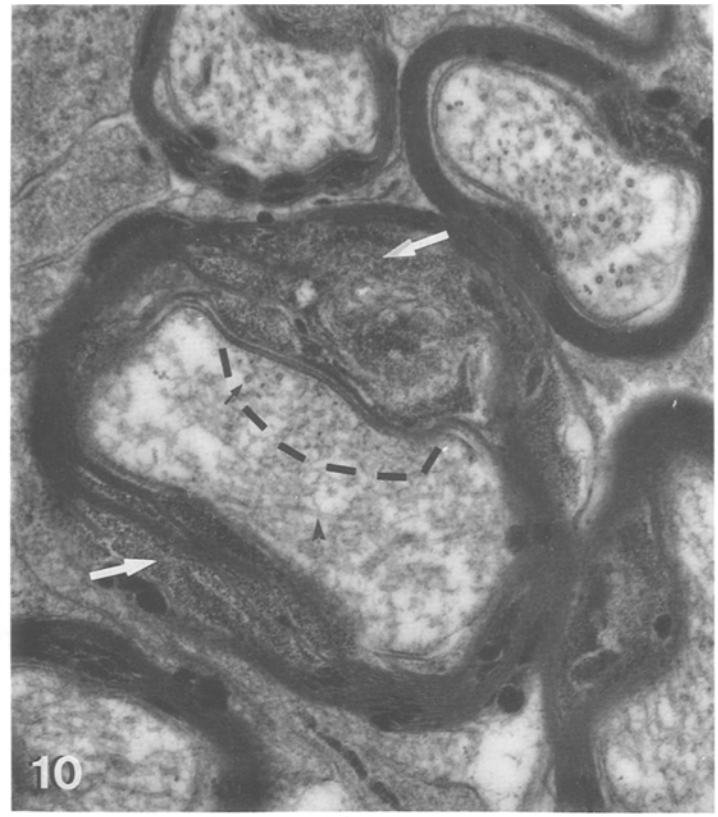
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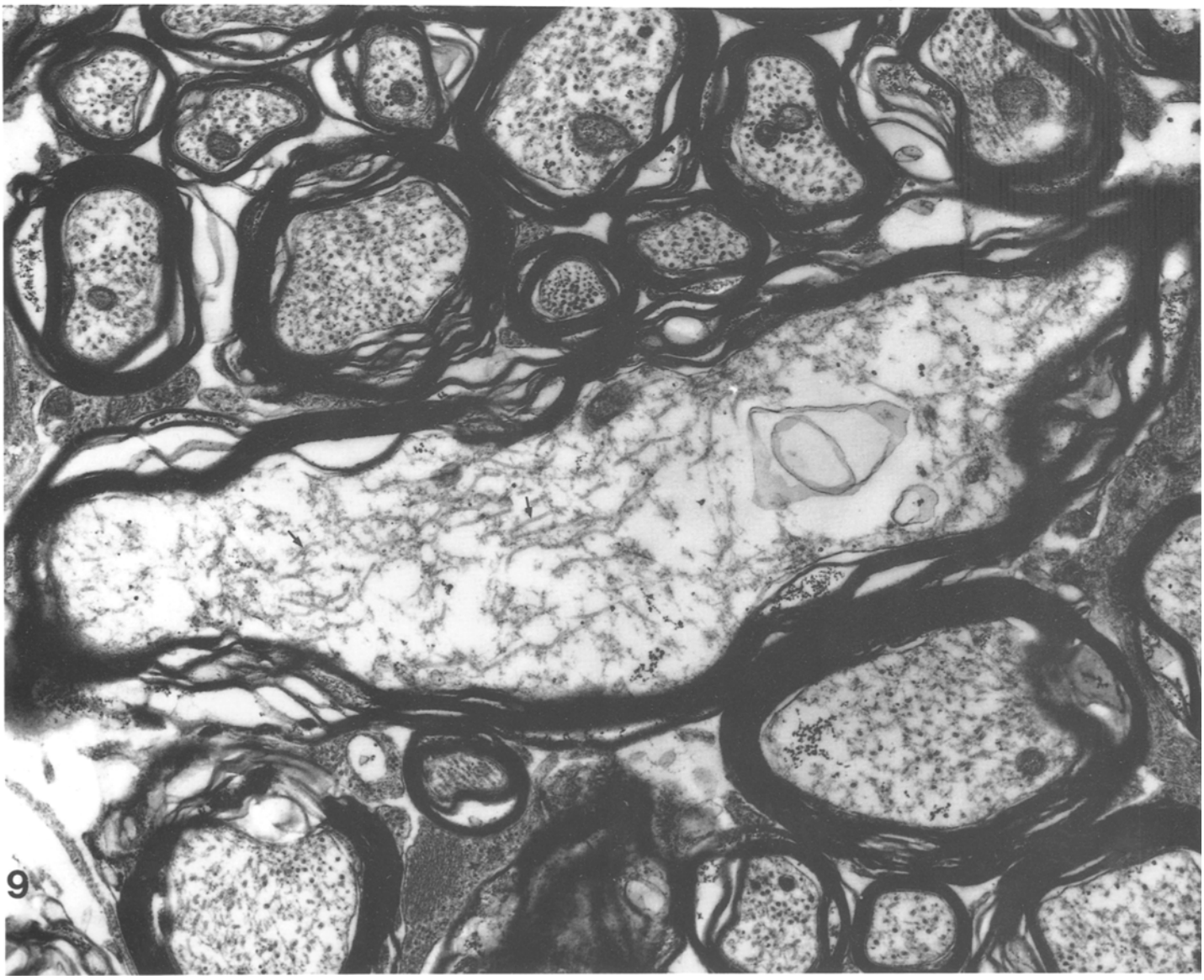
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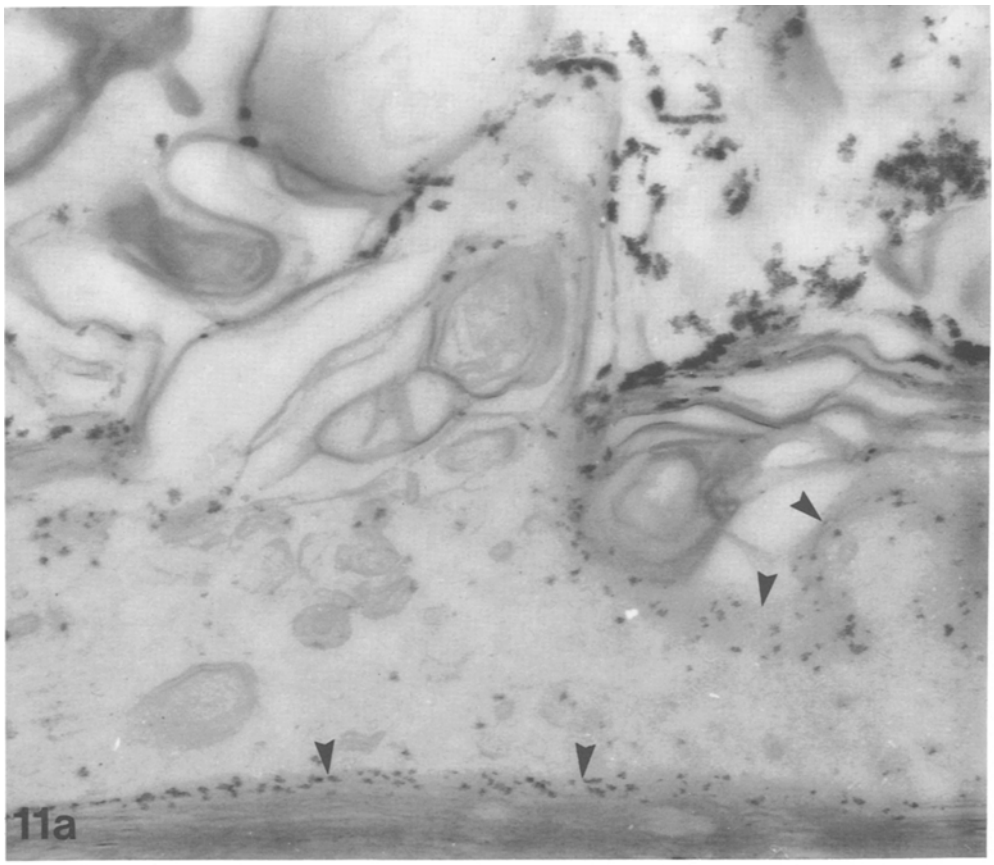

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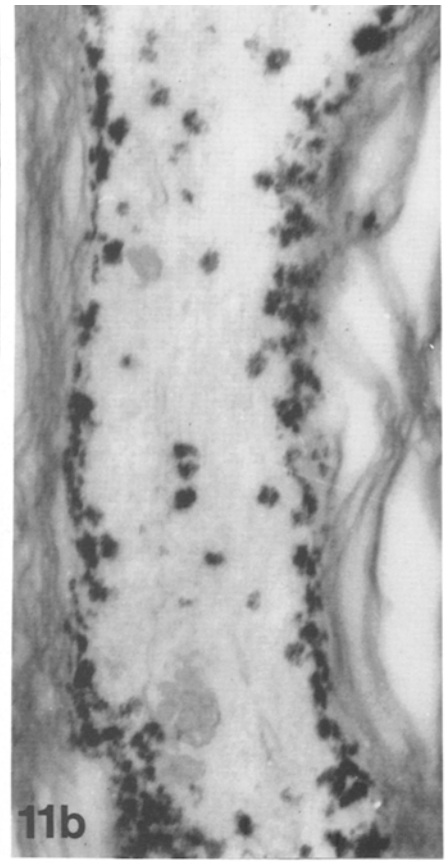

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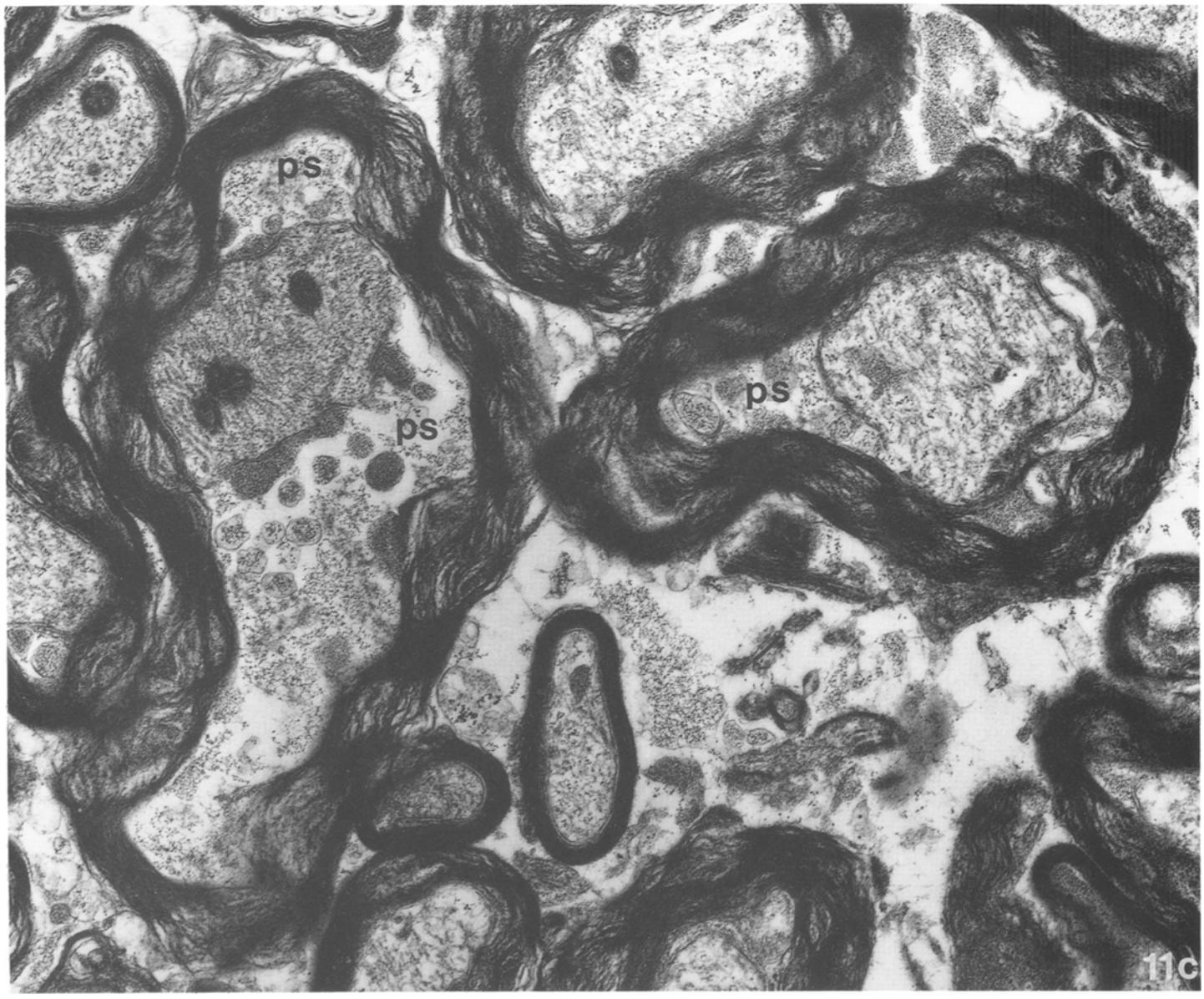

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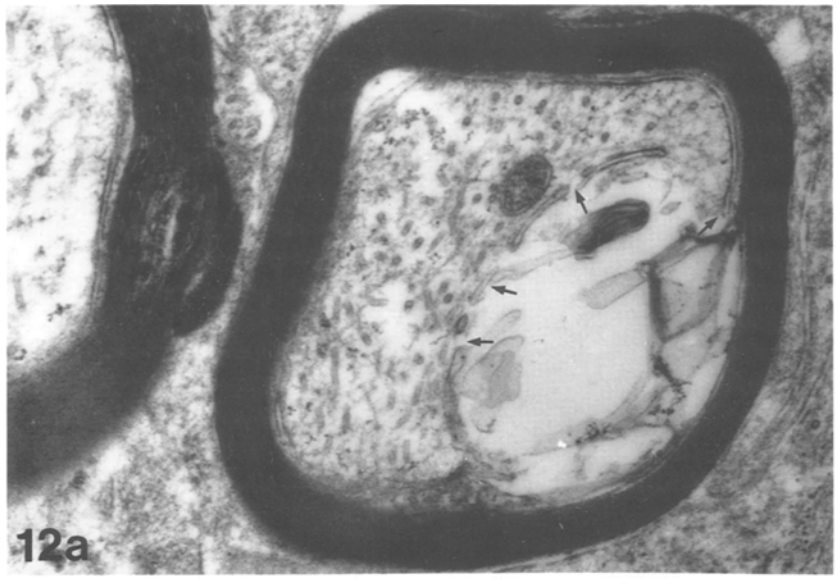

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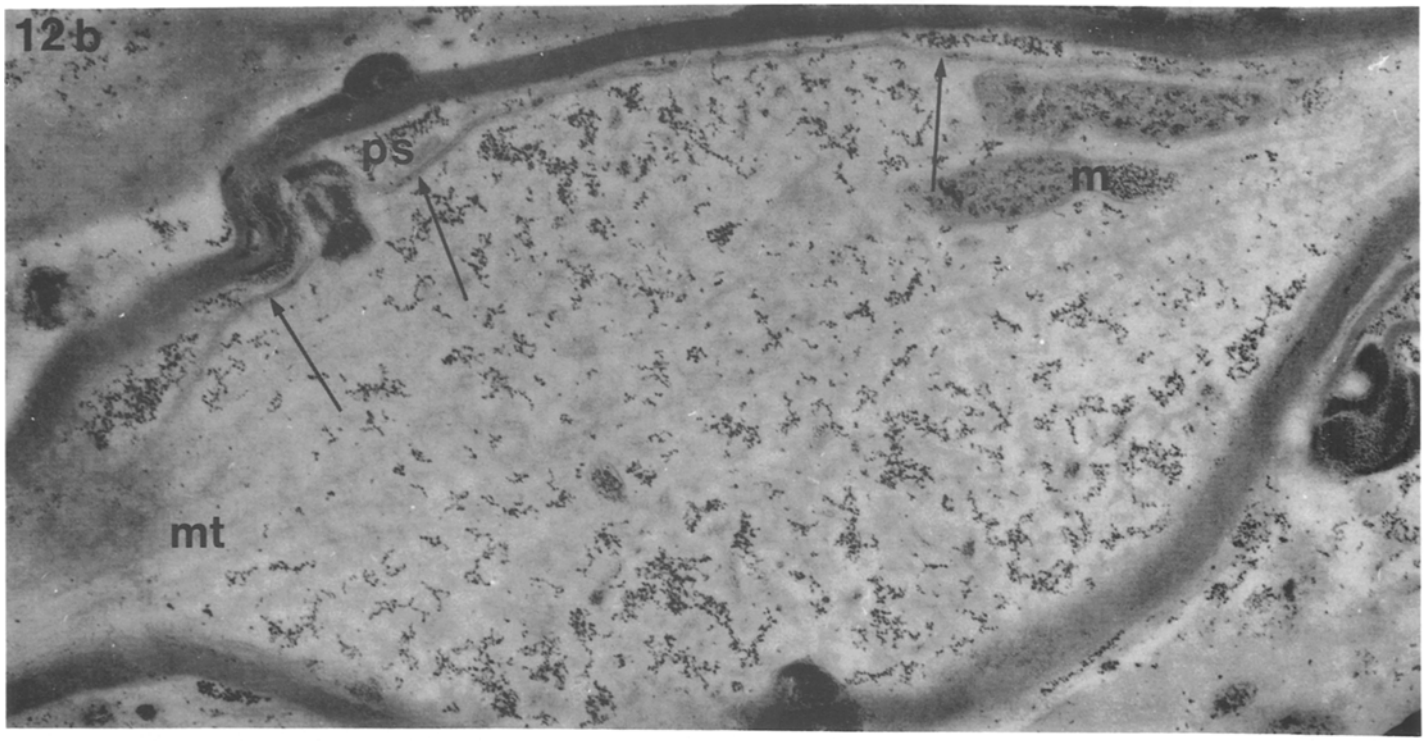

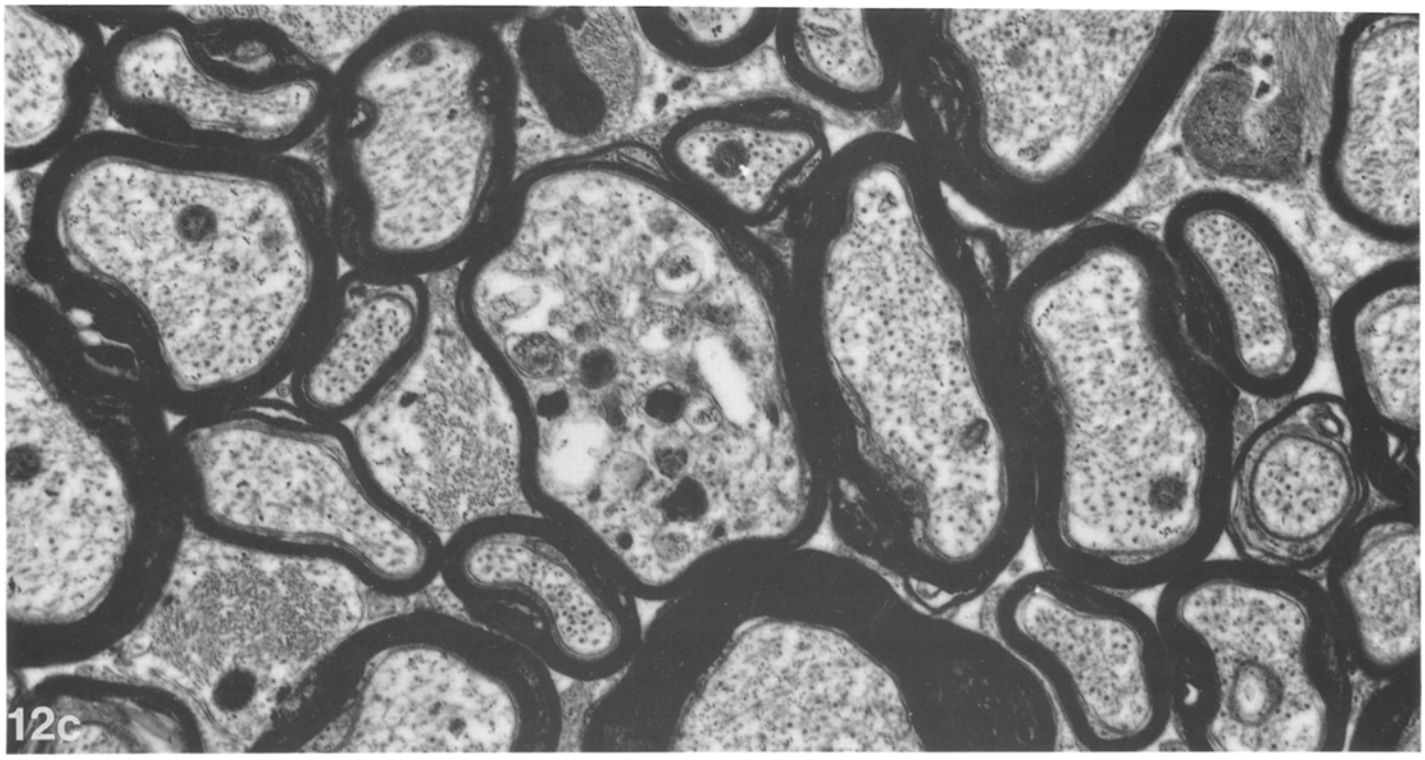

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comparable to that described above at 4 h. Periaxonal spaces were also frequent and contained aggregates of pyroantimonate precipitate.

We obtained morphological evidence in support of axotomy 24 h after injury. At this time there was complete disruption of any organized cytoskeleton within the remnants of myelin sheaths of damaged fibres (Fig. 14). There was separation of myelin lamellae with pyroantimonate precipitate distributed between the lamellae, or the myelin sheath was thinned around the area that it limited. No organized cytoskeleton was present (Fig. 14); rather there was a flocculent, amorphous ultrastructure and arrays of membranous profiles internal to the remnants of the myelin sheath. In other examples, the thinned myelin sheath limited an area of dense pyroantimonate precipitate (Fig. 15).

Discussion

We provide the first cytochemical evidence for the redistribution of calcium, Ca^{2+}-ATPase membrane pump and ecto-Ca-ATPase labelling in myelinated nerve fibres in the mammalian central nervous system after non-disruptive axonal injury. This paper extends observations noted earlier at nodes of Ranvier (Maxwell, 1995) and shows changes in the internodal region of injured axons and the myelin sheath. Focal aggregates of pyroantimonate precipitate occurred within nodes of Ranvier possessing 'nodal blebs'. Such nodes were most numerous 15 min after injury (Maxwell *et al.*, 1991b; Maxwell, 1995). Ecto-Ca^{2+}-ATPase activity was decreased as early as 15 min after injury at areas of dissociation or loosening of myelin lamellae. Pyroantimonate precipitate occurred in the areas of myelin dissociation. A novel finding was the occurrence of reaction product for membrane pump

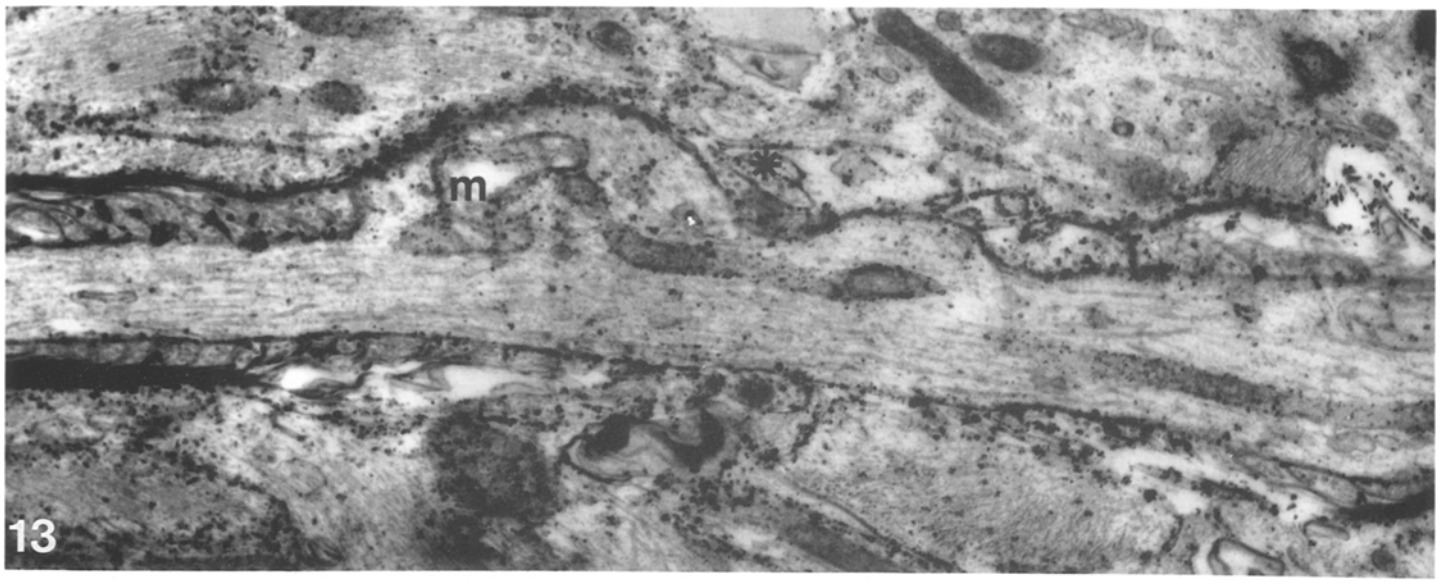
Fig. 8. Sections of myelinated fibres 1 h after stretch injury. The animal was processed for the pyroantimonate technique and sections examined unstained. The central fibre in the figure is cut in the transverse plane as indicated by the visualisation of myelin lamellae within the myelin sheath. There are numerous areas of separation of myelin lamellae and these areas contain pyroantimonate precipitate. At the upper edge of the fibre there is extension of a myelin intrusion (it) into the profile of the axon. The myelin intrusion is rich in pyroantimonate precipitate. A recognisable axonal cytoskeleton is only present in a small part of the axoplasm (arrowheads = neurofilaments, arrows = microtubules) ×57 500.

Fig. 9. Sections, examined unstained, of myelinated axons 2 h after stretch injury in material processed for the pyroantimonate technique. The majority of fibres are cut in transverse section and have an organised, recognisable axonal cytoskeleton despite some disruption of their myelin sheaths. But the fibre lying diagonally across the field possesses an electron lucent axoplasm which contains only small numbers of microtubules (arrows), together with some membranous profiles. ×28 000.

Fig. 10. A transverse section, examined unstained, of myelinated fibres 4 h after injury in material processed for the pyroantimonate technique. In the central fibre, the myelin sheath is disrupted and there are aggregates of pyroantimonate precipitate in the areas of disruption (white arrows). Only small numbers of neurofilaments (within the area delineated by the dotted line) and microtubules (black arrows) occur with the axoplasm the majority of which has a flocculent ultrastructure. Some microtubules (arrowheads) are no longer orientated parallel to the long axis of the axon. A discrete cytoskeleton is visible in an adjacent fibre (top right). ×40 000.

Fig. 11. Cytochemical localization of reaction product for membrane pump ATPase activity on the internodal axolemma. Material is taken (a) 1 h and (b) 4 h after stretch injury. Sections were incubated in 0.5 mM $CaCl_2$ and examined unstained. One hour after injury (a) there is light labelling on the irregularly profiled axolemma which is frequently retracted from the internal aspect of the disrupted myelin sheath (arrowheads). Within the axoplasm occur aggregates of membranous profiles, some of which are also labelled for ATPase activity. Four hours after injury (b), labelling for ATPase activity is heavier and is consistently localised on the axoplasmic face of the axolemma. (a) ×44 000; (b) ×36 000. In transverse, unstained, sections of axons 4 h after injury and processed for the pyroantimonate technique (c) there is widespread and marked disruption of the myelin sheath, particularly in the larger fibres in any one field. There are extensive periaxonal spaces (ps) in these fibres which contain concentrations of pyroantimonate precipitate. Adjacent, smaller fibres possess an intact myelin sheath which is closely related to the internodal axolemma. (c) ×29 000.

Fig. 12. Sections from optic nerves 4 h after stretch injury in material processed for the pyroantimonate technique and examined unstained. (a) A transverse section of a fibre in which the axon has an irregular profile and there are sites of discontinuity of the axolemma (arrows). (b) A longitudinal section of an axonal swelling illustrating a focal increase in axonal diameter in the swelling and the lesser calibre of the fibre on either side. Within the swelling the axolemma has an irregular profile (arrows) and is separated from the internal aspect of the myelin sheath to form a periaxonal space (ps). Pyroantimonate precipitate occurs within the periaxonal space. Two mitochondria occur within the axoplasm of the swelling and one of these (m) contains a concentration of pyroantimonate precipitate. There is still an indication of a linear organisation of the axonal cytoskeleton in the centre of the swelling (mt). There is, perhaps, a greater concentration of pyroantimonate precipitate in the peripheral region of the swelling. (a) ×40 500; (b) ×35 000. (c) Transverse sections of nerve fibres. The majority of fibres have a normal or close to normal ultrastructure with recognisable microtubules and neurofilaments within the axoplasm. But one fibre in the centre of the figure possesses a relatively thin myelin sheath and its axoplasm contains large numbers of membranous organelles, the majority of which contain aggregates of pyroantimonate precipitate. The axonal cytoskeleton in this axonal swelling is poorly organised. ×25 500.



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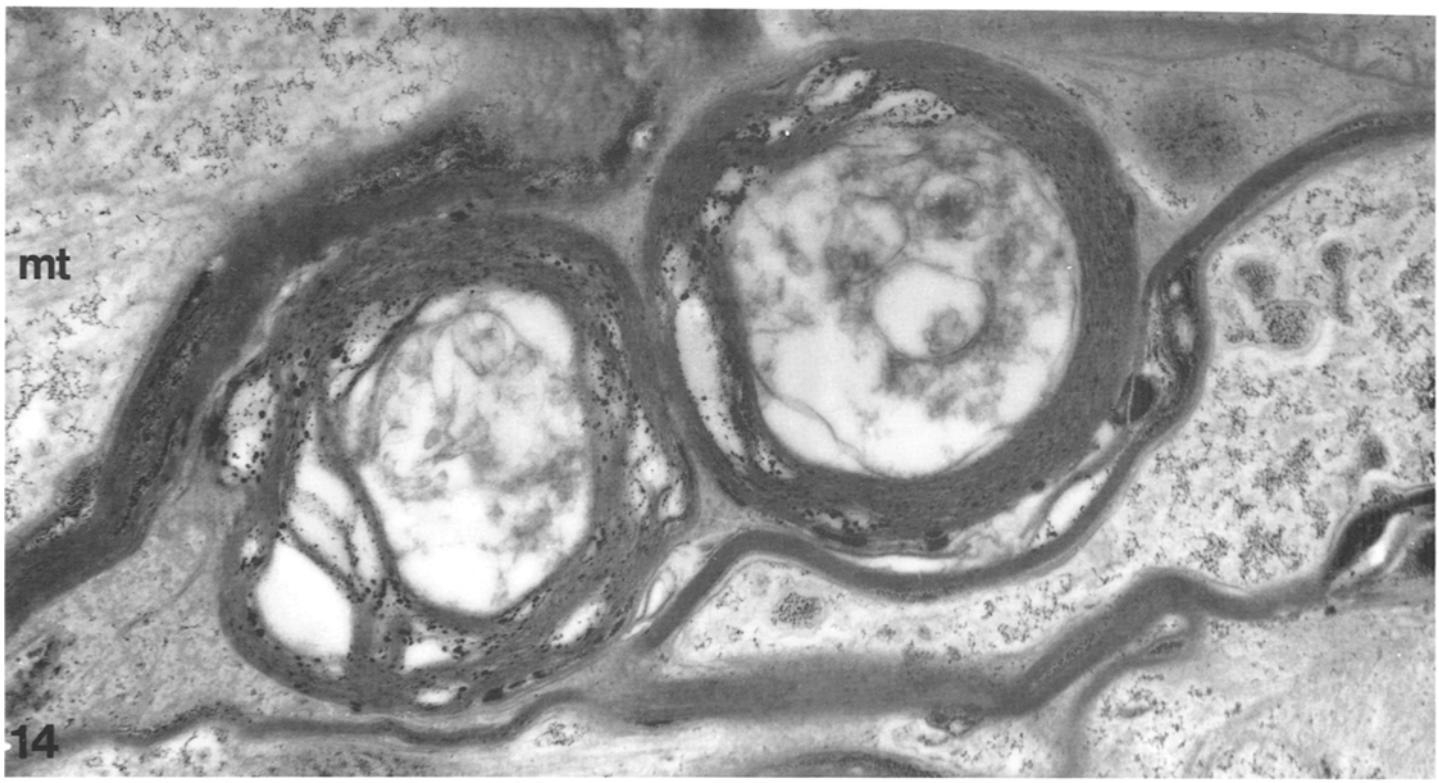

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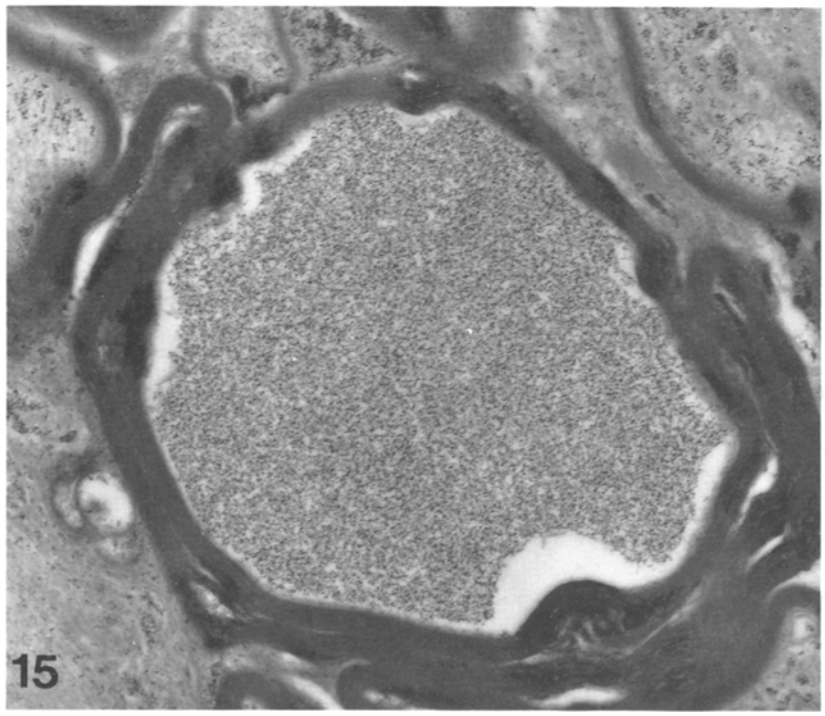

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Ca^{2+}-ATPase activity on the axoplasmic aspect of the internodal axolemma by 1 h, with an increased density of labelling 4 h after injury. At the same time there was loss, as demonstrated by loss of cytochemical labelling, of membrane pump Ca^{2+}-ATPase activity at the node of Ranvier. Disruption of the relationships of the myelin sheath and axolemma was more marked at longer survivals and was associated with concentrations of pyroantimonate precipitate and a loss of labelling for ecto-Ca^{2+}-ATPase activity. Myelin intrusions (Ochs & Jersild, 1990) occurred in nerve fibres at 1 h and later after stretch injury. Aggregates of pyroantimonate precipitate occurred in periaxonal spaces between the axolemma and inner aspect of the myelin sheath. The axolemma in these regions was characterized by infoldings into the axoplasm. Some of these infoldings contained myelin figures extending from myelin intrusions. These were associated with aggregates of pyroantimonate precipitate. We also obtained qualitative evidence for increased content of pyroantimonate within the axoplasm of nerve fibres, and complete disruption/dissolution of the axonal cytoskeleton by 4 h. At this time and at later survivals there was loss of axoplasm from within the myelin sheath of some fibres. We suggest that axotomy had occurred in these fibres within 4 and 6 h after non-disruptive axonal injury.

The specificity of the pyroantimonate technique was demonstrated by use of controls. Where pyroantimonate was deleted from the postfix or where sections were incubated in 10 mM EDTA the fine, diffuse precipitate was lacking. X-ray microanalysis of sections containing precipitate resulted in spectra with overlapping peaks for calcium and antimonate. These were lacking from sections devoid of precipitate. The precipitate was therefore demonstrated to be calcium pyroantimonate.

Recent evidence has indicated that membrane pump and ecto-Ca-ATPase activity may be distinguished by use of differential concentrations of $CaCl_2$ in the incubation medium during the cytochemical reaction (Kortje *et al.*, 1990; Barry, 1992) where labelling for membrane pump Ca^{2+}-ATPase activity may be obtained when the incubation medium contains 0.5–1 mM $CaCl_2$, while labelling for ecto-Ca-ATPase is obtained when $CaCl_2$ concentration is 5 mM and greater. Under the former conditions, the fact that labelling in our experimental material occurred predominantly on the axoplasmic face of the axolemma, and there was complete absence of labelling after fixation of tissue with glutaraldehyde provides further evidence for labelling of membrane pump Ca^{2+}-ATPase activity. Conversely, incubation in media containing 5 and 12 mM $CaCl_2$ in our experiments, that is at concentrations known to be inhibitory to membrane pump Ca^{2+}-ATPase activity (Barry, 1992), and where labelling occurred almost exclusively on the extracellular aspect of cell membranes provides evidence for labelling of ecto-Ca-ATPase activity. In addition, the technique that we have used in known not to cross-react with NA^{+}/K^{+}-ATPase because the distribution of the reaction product is not altered whether or not the reaction is carried out in the presence of ouabain (Mata *et al.*, 1989). There has been no previous attempt to demonstrate changes in either membrane pump or ecto-Ca^{2+}-ATPase activity in axons or their associated myelin sheath after either anoxia (Stys *et al.*, 1992) or traumatic brain injury. Since membrane pump Ca^{2+}-ATPase interacts with Ca^{2+} with high affinity and can reduce cytoplasmic concentrations of free calcium to extremely low levels (for review see Rega & Garrahan, 1986), it is suggested that alterations in membrane pump Ca^{2+}-ATPase activity are a better indicator of mechanisms to modulate influx of toxic levels of calcium into cells after injury than studies of the low affinity Na^{+}/Ca^{2+} exchanger system, except where activity of the ATPase is limited, for example, in hypoxic or ischaemic lesions. In addition, our data provides evidence in support of the hypothesis that dissociation of myelin lamellae, possibly correlated with loss of ecto-Ca-ATPase activity, may provide a route for calcium entry

Fig. 13. A longitudinal section of a node of Ranvier 24 h after stretch injury in material incubated in 1 mM $CaCl_2$ for ATPase activity and examined unstained. The node is elongated and the axolemma has an irregular profile. The nodal axoplasm contains a number of mitochondria, one of which (m) is swollen and electron lucent. The paranodal glial loops have a normal structure in the left hand paranode. But the glial loops are enlarged and electron lucent in the right paranode. A small group of dissociated glial loops (*) lie in relation to the axolemma. There is little evidence for cytochemical labelling on the axoplasmic aspect of the nodal axolemma. ×26 000.

Fig. 14. Sections of nerve fibres 24 h after stretch injury in material processed for the pyroantimonate technique and examined unstained. The remnants of the two central fibres contain no recognisable axon. Rather, within the myelin sheath there is a flocculent ultrastructure which contains fragments of membranous profiles. Closely adjacent fibres possess more recognisable axons. That on the left contains linearly organised groups of microtubules (mt) while that on the right contains aggregates of membranous profiles rich in pyroantimonate precipitate. ×35 000.

Fig. 15. A transverse section of the remnants of a nerve fibre 24 h after stretch injury from material processed for the pyroantimonate technique and examined unstained. Despite the presence of a recognisable myelin sheath, the axoplasm has been replaced by a dense concentration of pyroantimonate precipitate. The axon has been lost, or has undergone axotomy. ×25 000.

into stretch injured myelinated nerve fibres. However, we cannot totally discount the possibility that traumatic disruption of myelin in nerve fibres allows greater penetration by reagents and/or substrates which allow labelling of previously undemonstrable sites of ATPase activity.

Calcium influx into myelinated fibres of the sciatic nerve after crush injury (Mata *et al.*, 1986) has been demonstrated cytochemically. But no parallel investigation of alterations of either Ca^{2+}-ATPase membrane pump or ecto-Ca^{2+}-ATPase activity has been provided. The present paper provides the first cytochemical evidence for calcium influx after stretch/non-disruptive axonal injury, as defined in Maxwell and colleagues (1993), rather than a situation where axotomy occurs at the time of injury as in crush or transection. Non-disruptive axonal injury probably occurs in human head injuries ranging from mild (Blumbergs *et al.*, 1994) to severe (Gentleman *et al.*, 1995). Our cytochemical results suggest a lesser degree of acute axonal injury than that obtained after crush in that, although it is possible to demonstrate accumulation of pyroantimonate precipitate within swollen mitochondria, and the accumulation of vesicular organelles, both at nodes of Ranvier (Maxwell, 1995) and in internodal regions of axons, no evidence for fragmentation/vesiculization of the axoplasm at nodes, as described by Mata and colleagues (1986), or widening of the nodal gap through paranodal demyelination/retraction of the myelin sheath, within 4 h of stretch injury was obtained. However, we have now demonstrated the latter changes 24 h after stretch injury. This extends our earlier findings after lateral head acceleration in the non-human primate (Maxwell *et al.*, 1988) and may be interpreted as paranodal demyelination. But analysis of survivals ranging from 15 min to 24 h after stretch injury rather than a single time point of 4 h after crush (Mata *et al.*, 1986) allows us to demonstrate a progressive pathology in that there is a sequence of changes occurring over several hours after stretch/non-disruptive axonal injury resulting in 'secondary axotomy' (Maxwell *et al.*, 1993). The latter is manifested by the loss of an organized axonal cytoskeleton and the assumption of a flocculent ultrastructure several hours after the initial insult. Therefore, the present study provides the first cytochemical evidence for both calcium influx into stretch/non-disruptively injured myelinated fibres, and an altered labelling for membrane pump and ecto-Ca^{2+}-ATPase activities which, it is suggested, may contribute to the ensuing pathological cascade occurring in those fibres after such injury. Therefore the data provides strong, additional supporting evidence for a time course for axonal responses after non-disruptive axonal injury as has been suggested by other workers (reviewed by Maxwell *et al.*, 1991b; Povlishock, 1992; Pettus *et al.*, 1994). In addition, our studies, in conjunction with those at nodes of Ranvier (Maxwell, 1995), provide evidence in support of the suggestion that there are changes in axolemma and glial membrane structure and the activity of related membrane pumps and/or ecto-Ca-ATPases after stretch/non-disruptive axonal injury in normoxic nerve fibres. Our findings extend observations documenting membrane plasticity as indicated by loss of sodium channels from the axolemma in ethidium bromide/irradiated demyelinated dorsal column nerve fibres (Black *et al.*, 1991), loss of intramembranous particles from the nodal axolemma after stretch injury (Maxwell, 1995) and recent findings demonstrating altered axolemma permeability to large, tracer molecules such as horse-radish peroxidase (Pettus *et al.*, 1994). Our data extend the latter findings to demonstrate altered axolemmal permeability at the level of ionic moieties.

There are certain parallels between our results and those obtained by other workers in other models of axonal injury or pathology. Waxman and colleagues (1992) in their *in vitro* study of anoxic injury to the rat optic nerve demonstrated swollen mitochondria with a loss of cristae, dissolution of the axonal cytoskeleton with marked loss of microtubules and a lesser loss of neurofilaments together with the assumption of an amorphous ultrastructure, and the occurrence of large, so-called, intramyelinic spaces. The latter, we suggest, are equivalent to the periaxonal spaces that we describe after stretch injury, and which have also been described after contusion/weight drop injury to the spinal cord (Dohrman *et al.*, 1972; Ballentine, 1978). All of the morphological changes that we demonstrate parallel those described by Waxman and colleagues (1992) but with the major distinction that after stretch injury *in vivo* we can demonstrate a time course lasting several hours. Mitochondrial swelling occurs early and is followed by the development of periaxonal spaces and axolemma infolding. But loss of an organized axonal cytoskeleton is only observed at least 4 h after stretch injury, rather that after either 30 or 60 min of anoxia in the rat optic nerve (Webster & Ames, 1965; Waxman *et al.*, 1992), 30 min of cyanide inoxication in the rat corpus callosum (Hirano *et al.*, 1967), or after 30–240 min incubation of the desheathed sciatic nerve of the rat with the calcium ionophore A23187 (Schlaepfer, 1977).

However, our results differ from those of Waxman and colleagues (1992) in that dissociation/separation of myelin lamellae is not a notable feature in anoxic injury. Separation of myelin lamellae occurs within seconds of *in vitro* stretch injury in spinal nerve roots of the cat and sciatic nerves of the rat (Ochs & Jersild, 1990) and within 15 min of stretch injury to optic nerve fibres of the guinea-pig. It is probable that disruption of relationships of the myelin sheath occurs at the time of injury after the application of tensile strain to

myelinated optic nerve fibres. We posit that loss of ecto-Ca-ATPase activity at sites of myelin disruption allows influx of calcium into the myelin sheath, possibly mediating myelin dissociation, and into the periaxonal space. Our data provides evidence for a progressive increase in the extent of myelin sheath disruption and the development of periaxonal spaces over several hours after the initial insult to axons. In addition, our data provides further morphological evidence for a continuing and developing pathology in stretch injured myelinated nerve fibres. We argue that there may be a number of potential sites for calcium influx along the length of injured/damaged nerve fibres both at nodes of Ranvier (Maxwell, 1995) and in the internode.

The present study provides the first cytochemical evidence in support of the hypothesis (Adams *et al.*, 1991) that influx of calcium ions is a key factor in the development of axonal pathology after non-disruptive/stretch injury to mammalian, central, myelinated nerve fibres in a well characterized model for axonal injury (Gennarelli *et al.*, 1989; Maxwell *et al.*, 1991b). As discussed by Waxman and colleagues (1992), definitive demonstration of calcium influx into the axoplasm of injured axons will require use of ion-sensitive intracellular microelectrodes or ion-sensitive imaging. However, we argue, use of cytochemical techniques in this study provides evidence in support of calcium influx after non-disruptive axonal injury, possibly mediated by alterations in membrane associated ATPase activity. But definitive, quantitative data will only be provided by, for example, X-ray microanalysis of cryoquenched semithin sections (McCreath, 1993).

Mitochondrial swelling has been widely reported after a number of insults to central and peripheral nervous tissue, for example within 3 min of deprivation of oxygen and glucose (Webster & Ames, 1965), 12 min of fluid percussion traumatic brain injury (Pettus *et al.*, 1994), 15 min of stretch injury (the present paper), 60 min of anoxia (Waxman *et al.*, 1992), or 4 h after sciatic nerve crush (Mata *et al.*, 1986). Mitochondria do not normally accumulate much Ca^{2+} because their affinity for Ca^{2+} and the rate of its uptake is very low under normal physiological conditions (Scarpa, 1976) such that relatively low levels of pyroantimonate precipitate occur in mitochondria from control axons (Mata *et al.*, 1986; Maxwell *et al.*, 1991a). However, mitochondria sequester Ca^{2+} under pathological conditions (Schlaepfer, 1977), when the intracellular concentration exceeds about 5 μM (Blaustein, 1988). Cytochemical evidence for Ca^{2+} sequestration within swollen mitochondria has been provided after sciatic nerve crush injury (Mata *et al.*, 1986), in CNS fibres undergoing Wallerian degeneration (Wade *et al.*, 1980) and, in the current paper, after stretch injury to the guinea-pig optic nerve. Therefore we suggest that mitochondrial swelling noted in material fixed using routine techniques for transmission electron microscopy may be interpreted as morphological evidence for Ca^{2+} sequestration and reflects pathological levels of Ca^{2+} within lesioned or degenerating myelinated nerve fibres.

Perhaps the most exciting discovery in the present paper was the occurrence of reaction product indicating membrane pump Ca^{2+}-ATPase activity on the internodal axolemma at survivals greater than 1 h. Such labelling has never been found in normal or control material from the PNS/CNS where such labelling is restricted to the nodal axolemma (Mata *et al.*, 1988, 1989; Maxwell *et al.*, 1991a), although it should be borne in mind that the cytochemical techniques used in those papers may not have been totally appropriate. In the present paper, we argue that our results demonstrate labelling for membrane pump Ca^{2+}-ATPase activity for two reasons. First, that reaction product occurred on the axoplasmic rather than the extracellular aspect of the axolemma. The ATP binding site for calcium pump activity is situated on the cytoplasmic side of the plasmamembrane (Akisaka & Oda, 1977; Ando *et al.*, 1981; Penniston, 1983; Mughal *et al.*, 1989; Kortje *et al.*, 1990; Gioglio *et al.*, 1991; Pappas & Kriho, 1991; Barry, 1992). Second, that the concentration of $CaCl_2$ that we utilized in some of our incubation media favoured labelling for cytoplasmic Ca^{2+}-ATPases. Labelling for Ca^{2+}-ATPase activity in the internodal axolemma after stretch/non-disruptive axonal injury may indicate either an unmasking of sites of activity mediated by increased penetration of reagents where there is separation of myelin lamellae, or the insertion of new membrane containing ATPase activity. We suggest that the time course that we report here, that is hours after injury, makes it unlikely that new membrane may be axonally transported from the cell soma. Thus the source of membrane pump activity documented here for the first time requires further investigation before firm conclusions can be drawn. Nonetheless, the present findings may indicate an attempt by the axon to regain Ca^{2+} homeostasis either when ecto-Ca-ATPase activity has been lost from the myelin sheath or there is reversed activity of the Na^+/Ca^{2+} exchanger. We provide evidence for the former in the present paper but the latter has not yet been demonstrated after stretch injury to axons. Reversed activity of the Na^+/Ca^{2+} exchanger does, however, occur during cell depolarization (Blaustein, 1988) and/or anoxia/ischaemia (Stys *et al.*, 1992). Despite the fact that Tomei and colleagues (1990) provided electrophysiological evidence for a reduced number of active axons and a lowering of their conduction velocity after stretch injury to the optic nerve of adult guinea-pigs, there is currently no information concerning levels of Na^+/Ca^{2+} activity after stretch injury to central, myelinated nerve fibres

and therefore the contribution made to calcium influx by reversed activity of the Na^+/Ca^{2+} exchanger during posttraumatic depolarisation, but in normoxic animals, cannot presently be estimated. This phenomenon requires further investigation. The present demonstration of altered pyroantimonate distribution in stretch injured myelinated fibres and of altered distribution of both membrane pump and ecto-Ca^{2+}-ATPase activity provides the first evidence in support of the hypothesized (Adams *et al.*, 1991) influx of calcium ions into stretch injured nerve fibres. Our findings provide the first cytochemical evidence for calcium influx into damaged nerve fibres in a model of human diffuse brain injury and that this influx may be mediated, at least in part, by changes in or loss of membrane pump activity. We argue that changes in cytochemical labelling of the internodal axolemma might, possibly, reflect an attempt by the damaged axon to regain Ca^{2+} homeostasis.

Acknowledgments

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