Cross-linker System between Neurofilaments, Microtubules, and Membranous Organelles in Frog Axons Revealed by the Quick-freeze, Deep-etching Method

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ABSTRACT   The elaborate cross-connections among membranous organelles (MO), microtubules (MT), and neurofilaments (NF) were demonstrated in unfixed axons by the quick-freeze, deep-etch, and rotary-shadowing method. They were categorized into three groups: NF-associated cross-linkers, MT-associated cross-bridges, and long cross-links in the subaxolemmal space. Other methods were also employed to make sure that the observed cross-connections in the unfixed axons were not a result of artifactual condensation or precipitation of soluble components or salt during deep-etching. Axolemma were permeabilized either chemically (0.1% saponin) or physically (gentle homogenization), to allow egress of their soluble components from the axon; or else the axons were washed with distilled water after fixation. After physical rupture of the axolemma or saponin treatment, most of the MO remained intact. MT were stabilized by adding taxol in the incubation medium.

Axons prepared by these methods contained many longitudinally oriented NF connected to each other by numerous fine cross-linkers (4-6 nm in diameter, 20-50 nm in length). Two specialized regions were apparent within the axons: one composed of fascicles of MT linked with each other by fine cross-bridges; the other was in the subaxolemmal space and consisted of actinlike filaments and a network of long cross-links (50-150 nm) which connected axolemma and actinlike filaments with NF and MT. F-actin was localized to the subaxolemmal space by the nitrobenzooxadiazol phallacidin method. MO were located mainly in these two specialized regions and were initmately associated with MT via fine short (10-20 nm in length) cross-bridges. Cross-links from NF to MO and MT were also common. All these cross-connections were observed after chemical extraction or physical rupture of the axon; however, these procedures removed granular materials which were attached to the filaments in the fresh unextracted axons. The cross-connections were also found in the axons washed with distilled water after fixation. I conclude that the cross-connections are real structures while the granular material is composed of soluble material, probably protein in nature.

The inability of the axon to synthesize proteins means that substances manufactured in the cell body must be transported from the cell body to the nerve terminals. Certain characteristics of this transport system have already been identified. Light microscope cinematography studies (3, 6, 12) clearly demonstrated anterograde and retrograde saltatory movement of membranous organelles (MO) in the axon. Accumulation of MO at the proximal and distal sides of ligated axons has been observed by electron microscopy (EM) (44, 45). A number of biochemical and autoradiographical studies have revealed that at least five different groups of materials move down the axons at different transport velocities (2, 7, 13, 14, 16, 24, 46, 47, 48, 50). Several different models of axonal transport involving neurofilaments (NF) and microtubules (MT) have been proposed, but the precise mechanisms are still unknown (8, 9, 11, 17, 37). Detailed structural analysis of the cytoskeleton of the axon is required for a sound basis on which to build such models.

Thin section studies have shown that longitudinally oriented NF and MT are the main constituents of the axon (4, 25, 27,

30, 51). They also revealed lateral arms b~'ween MT and some of the MO (42, 43). In addition, a suba.. .emmal filamentous network has been demonstrated by scanning EM (SEM) (31). Recently, Ellisman and Porter (10) studied the cytoskeleton of the axoplasmic matrix by a number of techniques including high voltage EM of thick sections, transmission EM, and SEM of embedding medium-free thin sections (polyethylene glycol [PEG] method), and also quick-freezing and deep-etching of fixed tissues. They observed a three-dimensional lattice in the axoplasm and proposed its active involvement in organelle transport. In an effort to further extend the resolution and to reduce the possibility of artifacts caused by chemical fixation (29, 41), I studied the structure of axoplasm in unfixed tissues by the method of quick-freezing and deep-etching (20, 21). In addition, I undertook several different procedures to circumvent possible artifacts caused by deep-etching. These studies clearly revealed the three-dimensional architecture of the axonal cytoskeleton and especially demonstrate the fine cross-connections between MT, NF, and MO. Particularly important is the observation that MO (tubular membrane structures, vesicles of various sizes, and mitochondria) were located in the columns delineated by MT and were connected to MT by fine cross-bridges. This morphology is consistent with the idea that the MT work as rails or tracks for the movement of MO and that cross-bridges could play an active role in that process. However, the relative importance of cross-links for functional vs. structural roles awaits further study of the chemical nature.

MATERIALS AND METHODS

Fresh Axons

Very thin nerves from segments IV, V, and VI of the spinal cord of small *Rana pipiens* (body length 1-1.5 in) were tied at both ends with silk threads, dissected out, and quick-frozen.

Chemical Permeabilization of the Axolemma

Spinal nerves of small *R. pipiens* were tied at both ends and pinned down in Sylgard bottomed petri dishes containing physiological frog saline (116 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 0.5 mM NaH₂PO₄, and 5 mM HEPES buffer, pH 7.0, with 5% glucose added). They were washed with Ca⁺⁺-free saline (containing 5 mM MgCl₂, 3 mM EGTA instead of CaCl₂) twice and were incubated with 0.1% saponin in 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA 30 mM HEPES, 10 µM taxol (39), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 20 min at room temperature. After a brief washing with the same buffer without saponin, some of the samples were quick-frozen and others were fixed with 1% paraformaldehyde, 70 mM KCl, 5 mM MgCl₂, 3 mM EGTA, 30 mM HEPES, 10 µM taxol for 1.5 h, briefly washed with distilled water and quick-frozen.

Physical Rupture of the Axolemma

Torpedo omata were decapitated and the electric organ was dissected out. It was cut into small pieces and gently homogenized in 70 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES, 0.1 mM PMSF, 10 µM taxol, pH 7.2, with five strokes in a Dounce homogenizer with a Teflon pestle rotating at 50 rpm at room temperature. The homogenate was centrifuged for 3 min at 3,000 g and the pellet

was washed twice with the same buffer. Then the pellet was quick-frozen. Some pellets were fixed with 1% paraformaldehyde in the same buffer mentioned above for 1.5 h, washed with distilled water and quick-frozen.

Fixed Axons

To examine the effects of chemical fixation, the following experiments were carried out: (a) Fixation with glutaraldehyde and paraformaldehyde: Frog spinal nerves were tied and dissected and pinned down on Sylgard-coated petri dishes. They were fixed with 1% glutaraldehyde, 2% paraformaldehyde, 115 mM NaCl, 2 mM CaCl₂, 30 mM HEPES, pH 7.4, for 1.5 h. Some of them were directly quick-frozen and others were washed extensively with distilled water before freezing. (b) Fixation with aldehyde and osmium tetroxide: Frog spinal nerves were fixed with aldehyde as mentioned above. After a brief washing with barbital buffer, the nerves were postfixed with 1% OsO₄ in barbital buffer, pH 7.0, for 1 h at 4°C. Then they were washed with distilled water and quick-frozen.

Quick-freezing, Freeze-fracture, and Electron Microscopy

All samples were frozen by contact with a pure copper block cooled by liquid helium as previously described (20). The very surfaces (<10 µm) of the frozen samples were fractured in a Balzers 400 (Balzers, Hudson, NH) at −196°C at a vacuum of <2-5 × 10⁻⁷ Torr and etched for 4 min at −95°C. Then they were recooled to −150°C and replicated by rotary shadowing with platinum and carbon. Tissues were dissolved in chromic sulfuric acid or Purex (Purex Corp., Lakewood, CA) and replicas were cleaned with distilled water and picked up on Formvar-coated grids. Stereo micrographs were taken by a JEOL 100 CX or 200 CX electron microscope at 100 KV at ±10° tilt.

Cytochemistry of Localization of F-actin in the Axon by Nitrobenzooxadiazol-labeled Phallacidin

Sciatic nerves were dissected out from *R. pipiens*. They were fixed with 1% paraformaldehyde, 115 mM NaCl, 5 mM MgCl₂, 2 mM EGTA, 30 mM HEPES, pH 7.4, for 30 min. They were frozen in liquid freon and cut into 4-6 nm thick longitudinal or cross sections in a DAMON Cryostat. They were collected on slide glasses and incubated in a drop of 5 U of nitrobenzooxadiazol (NBD)-phallacidin/ml (Molecular Probes Inc., Plano, TX), 100 mg lysophosphatidylcholine/ml, 70 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 20 mM HEPES, pH 7.4, for 20 min at room temperature. Then they were washed twice with 70 mM KCl, 5 mM MgCl₂, 2 mM EGTA, 20 mM HEPES, pH 7.4, and mounted with 50% glycerol.

The samples were observed with a Zeiss fluorescence microscope.

RESULTS

Overall Architecture of Axoplasm in Fresh Axons

Most of the axonal matrix was filled with longitudinally oriented NF, 11 nm in diameter, which were cross-linked with each other by numerous fine short fibrils. However, since the dimensions of the structures mentioned in this paper include a metal coat, true diameters could be ~1 nm less. Within this lattice of NF two types of specialized regions were recognized. One was fascicles of MT (25 nm in diameter) which were linked with each other by fine shorter cross-bridges (Fig. 1). MT sometimes appeared to form columns (Fig. 1). Another was the subaxolemmal space which consisted of a dense net-

FIGURE 1 Untreated quick-frozen fresh frog axon. This micrograph shows the columns delineated by MT (thick arrows) 25 nm in diameter displaying protofilament structures on the surface. NF (thin arrows) 11 nm in diameter are also found to run longitudinally. Numerous MO including vesicles of various sizes, tubular membrane structures, and mitochondria are observed in between the fascicles of MT. Short thin cross-bridges connect these MO to MT (arrowheads on the mitochondria [M] and arrows in the *insert*). The MO are also cross-linked with NF (*insert*, arrowheads). Many granular materials are attached to the NF, MT, cross-linkers, and the surface of MO. Bar 0.1 µm, × 127,000. *Inset* shows an MO (not a mitochondria judged by the few P face particles) closely associated with MT. Between them cross-bridges are observed. Bar 0.1 µm. × 204,000.
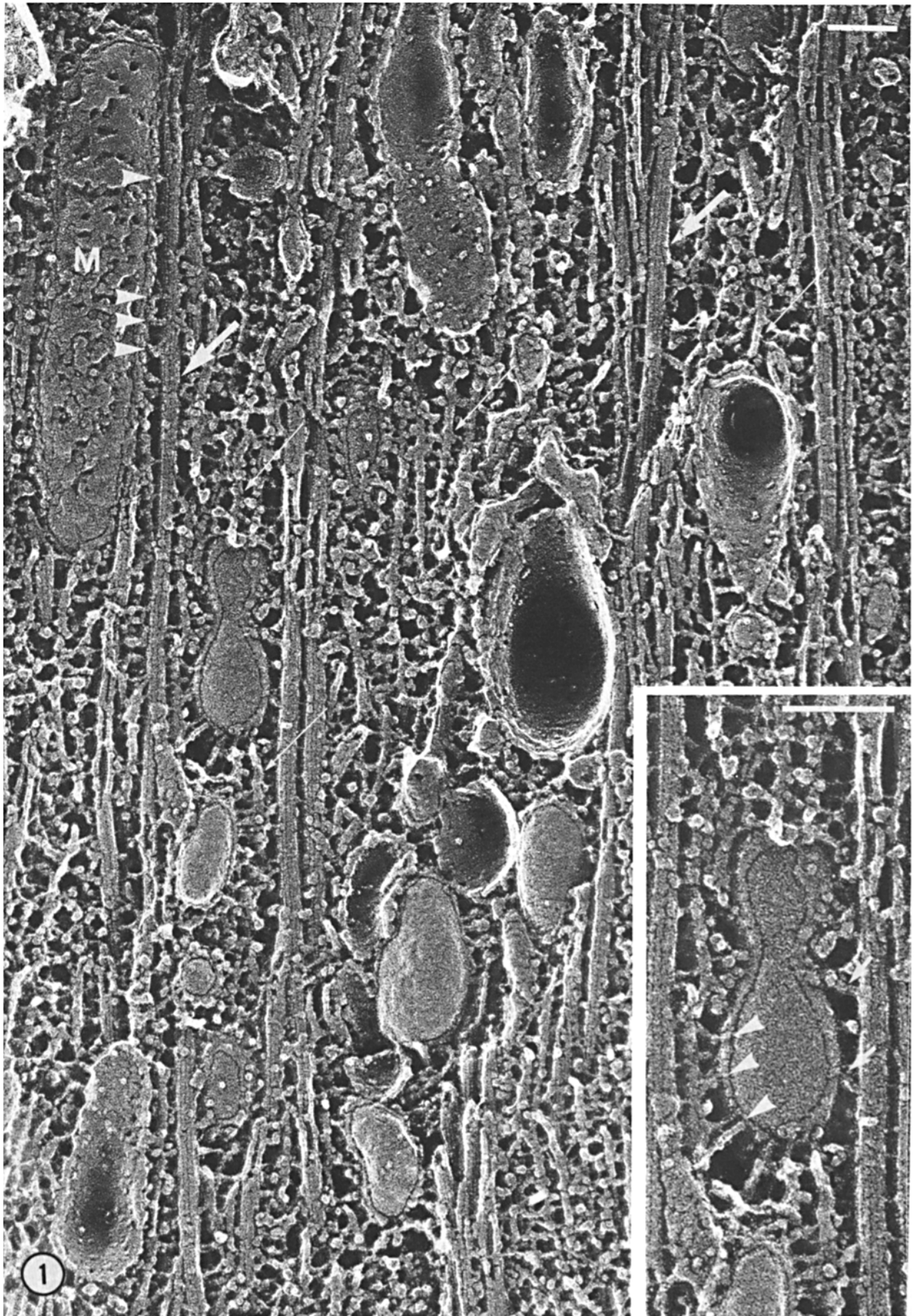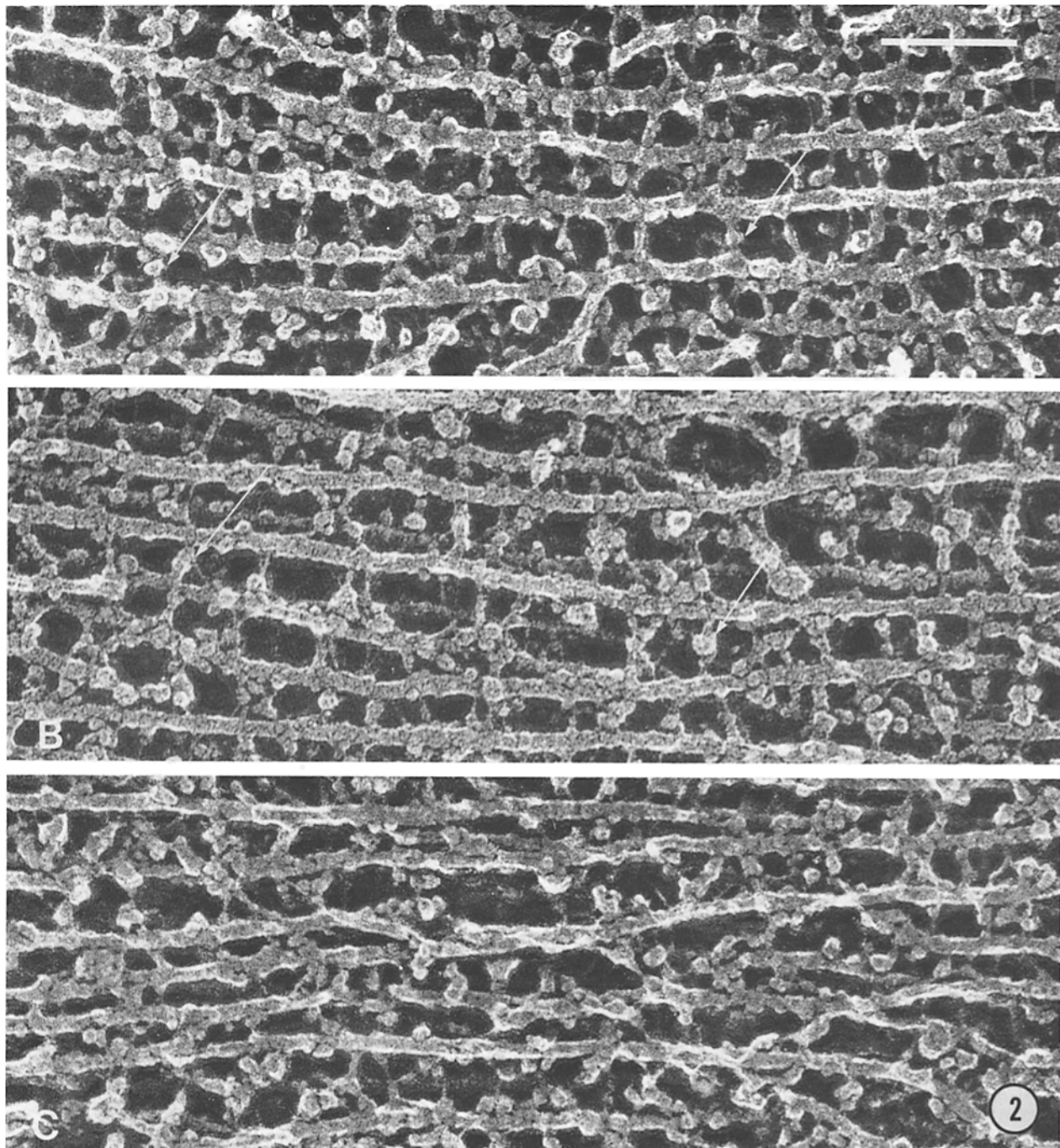
work of longer cross-links connecting axolemma to MT and NF (see Fig. 8A). MO such as tubular membrane structures, vesicles of various sizes, and mitochondria were predominantly localized in these specialized regions (Fig. 1). These organelles tended to be closely associated with MT and were very often interconnected by fine cross-bridges (see Figs. 1, 5A, and 7A, B, E, F, and G). Cross-links were also frequently found between NF and MO (see Figs. 1 and 5A). In fresh samples granular materials were seen on the surfaces of MO, MT, NF, and cross-connections between these elements.

The following experiments were performed to investigate the possibility that the observed structures in fresh samples were artifacts of condensation and precipitation of salt or soluble components of axoplasm.

Comparison of the Cross-connections in the Axon after Various Methods of Tissue Preparation

The NF lattice is the simplest structure in which to compare the structural changes after various treatments. In the unfixed axon NF, 11 nm in diameter, were connected by numerous cross-linkers 4–6 nm in diameter 20–50 nm long. A characteristic of the fresh sample was that the surfaces of both NF and cross-linkers were decorated with occasional granular materials. Thus, the cross-linkers appeared knobby (Fig. 2A). The NF and cross-linkers in the axons, which were fixed with aldehyde and washed extensively with distilled water to remove salt before freezing, appeared to be the same as those in the
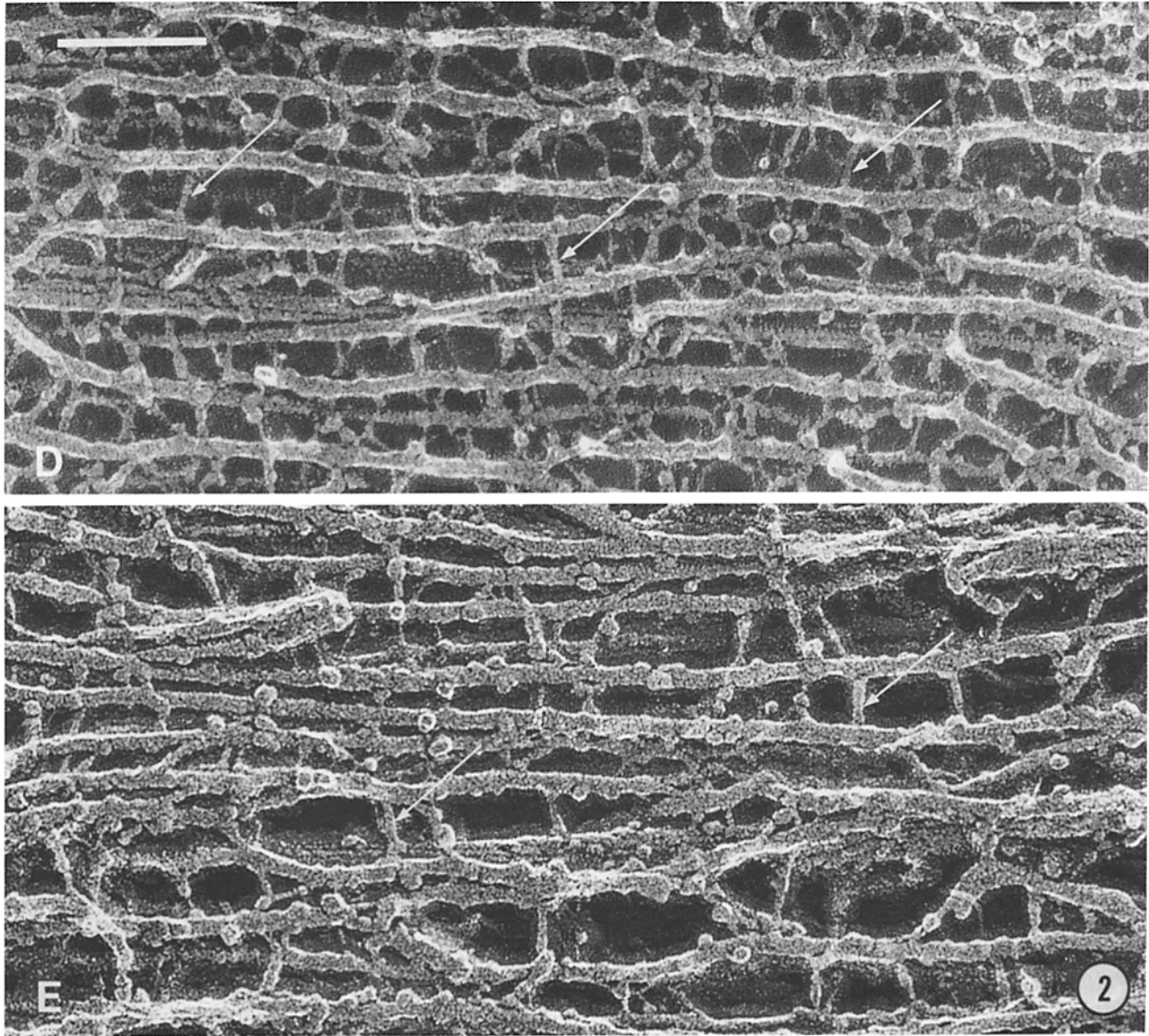
FIGURE 2 Comparison of quick-frozen, deeply etched NF after various treatments. (A) Fresh frog axon. This micrograph shows the parallel array of NF. The space between adjacent NF varies in width between 20 to 50 nm. Cross-linking thin filaments (4-6 nm in diameter) connect adjacent NF. Granular materials are very often attached to the NF and cross-linkers (arrows). (B) Frog axon fixed with glutaraldehyde and paraformaldehyde, washed with distilled water and quick-frozen. NF and cross-linkers resemble those in the unfixed samples. Granular materials decorating NF and cross-linkers (arrows) are prominent. (C) Frog axon fixed with aldehyde followed by osmification (1% OsO₄ at 4°C for 1 h), washed with distilled water, and quick-frozen. Although cross-linkers exist, they are fewer in number than in A or B. (D) Frog axon incubated with 0.1% saponin for 20 min before freezing. The cross-linkers certainly remain after saponin treatment, but granular materials are removed and the cross-linkers appear more uniform in diameter (arrows). The number of the cross-linkers is about same as in the fresh axon. (E) Torpedo axon whose axolemma was partially broken by gentle homogenization before freezing. The cross-linkers are fewer in number than in the saponin treated axons (probably because some of them are broken in the process of homogenization), but they (arrows) look quite clear and uniform in diameter. Knobby structures attached to the NF seem to be broken cross-linkers in this case. Bars, 0.1 μm. × 214,000.

fresh samples (Fig. 2B). Furthermore, the samples fixed with aldehyde and quick-frozen directly without washing with distilled water showed no detectable differences compared with those washed with distilled water before freezing (data not shown). However, when aldehyde-fixed samples were postfixed with OsO₄, washed with distilled water and quick-frozen, it was noticed that the cross-linkers decreased in number and were somewhat distorted (Fig. 2C).

To check the possibility that soluble components in the axoplasm could still be condensed during the process of fixation, freezing, or deep-etching in the fixed or fresh samples, the

axolemma was permeabilized chemically or physically before freezing to allow egress of soluble proteins.

After chemically perforating the axolemma with saponin, most of the granular material on the surface of the NF and cross-linkers was removed (Fig. 2D). As a result, cross-linkers appeared to be relatively uniform in diameter (4-6 nm) and were as numerous as in fresh axons (Fig. 2D). This was confirmed by disrupting the axolemma physically through gentle homogenization before freezing; in this case some of the cross-linkers were broken and appeared as knobs on the NF (Fig. 2E). A summary follows of the cytoskeletal structure of
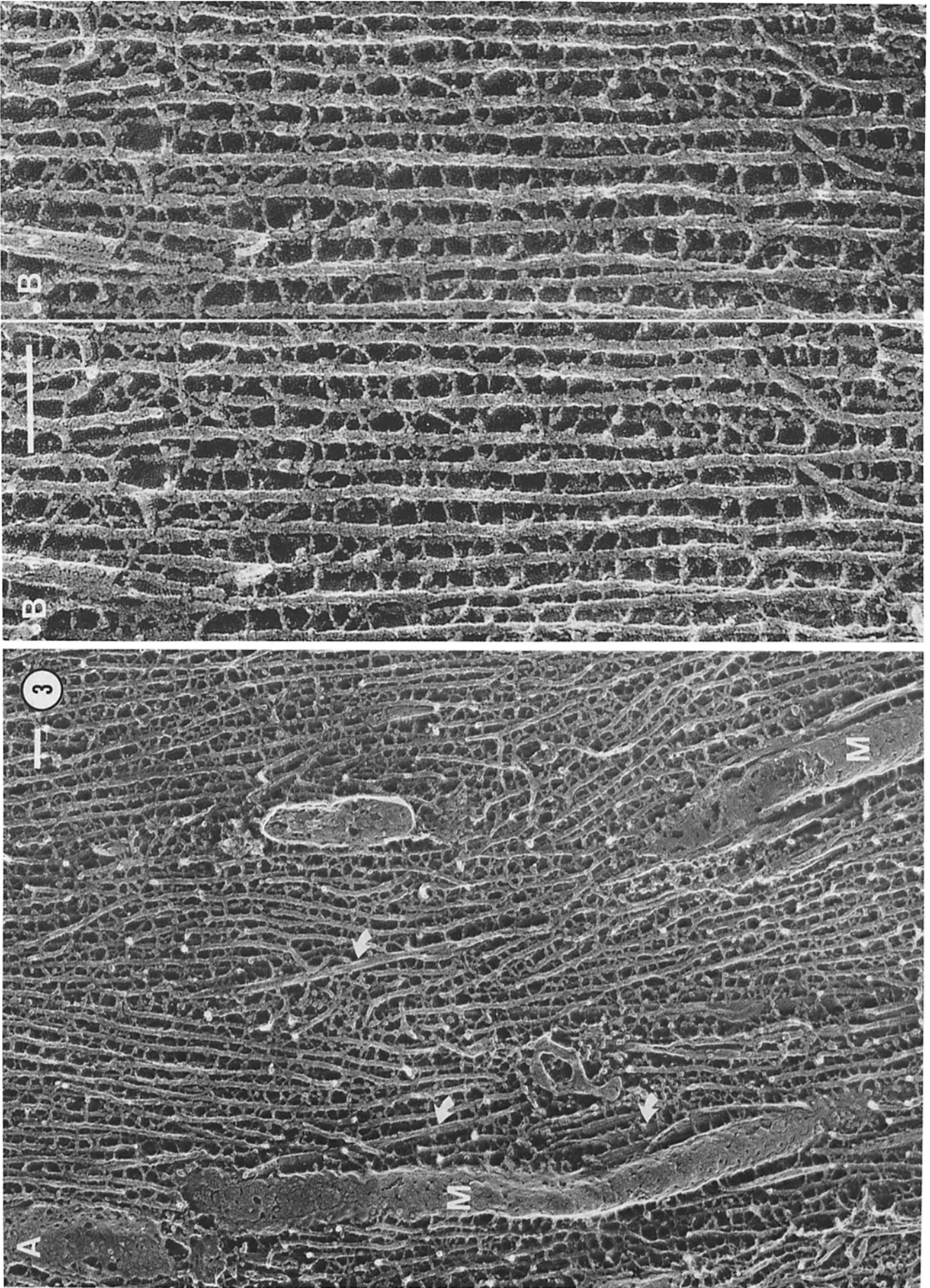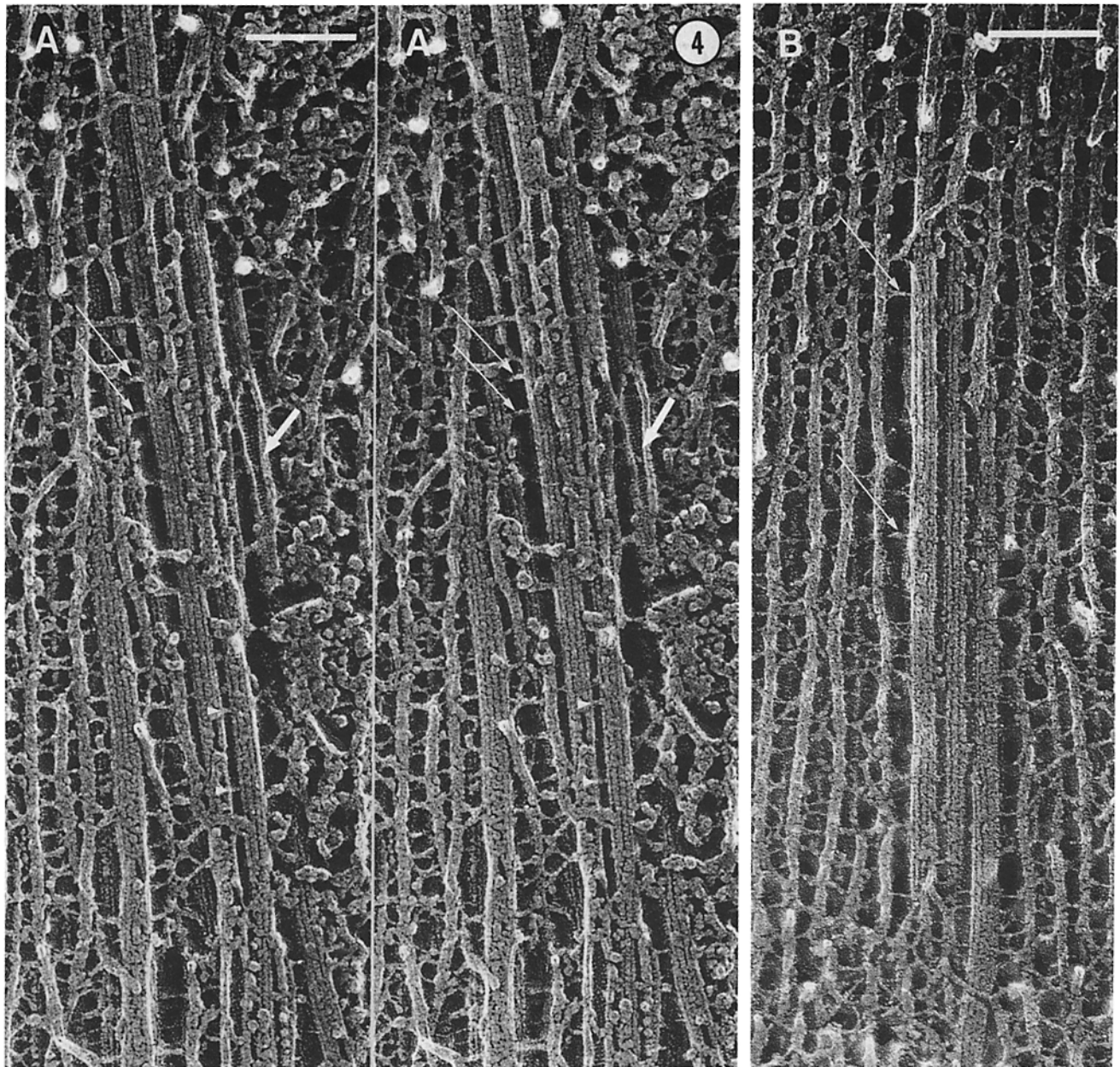
FIGURE 4 (A) This micrograph shows a stereo pair of MT bundles in a saponin-treated frog axon. MT were stabilized by taxol in the incubation medium. MT tend to run as a group. The outer surface of the MT is characterized by longitudinal substructures showing an arrangement of protofilaments and at the inner surface (short arrows) regular 4-nm oblique stripes representing a staggered arrangement of protofilaments are found. Sometimes cross-bridges (4–6 nm in diameter) (long arrows and arrowheads) are connecting adjacent MT. They are fewer in number and shorter than cross-linkers between NF. Arrows point to the longest cross-bridges but they are usually much shorter like those indicated by the arrowheads. Bar, 0.1 μm. × 179,000. (B) MT bundles in a saponin treated frog axon. Three MT exist adjacent to the NF. Very often, cross-linkers (arrows) (4–6 nm in diameter, 20–30 nm in length) connect the MT almost perpendicularly to the NF. Bar, 0.1 μm. × 179,000.

FIGURE 3 (A) This micrograph shows a low magnification view of a saponin-treated frog axon. Interestingly, the saponin did not affect mitochondrial membrane (M). Axoplasm is filled with numerous longitudinally oriented NF between which evident thin short cross-linkers exist. Fascicles of MT (arrows) exist in between the NF. MT are closely associated with mitochondria (M). Note that most of the granular materials are removed and the cytoplasm looks clearer compared with fresh axon. Bar, 0.1 μm. × 71,000. (B) A stereo pair of NF (11 nm in diameter) run parallel with each other (in this micrograph ~20–30 nm apart). Numerous cross-linkers connect adjacent NF, mostly perpendicularly, like ladders. The cross-linkers are ~4–6 nm in diameter 20–30 nm in length in this micrograph (sometimes much longer). Granular materials are almost removed. Bar, 0.1 μm. × 203,000.
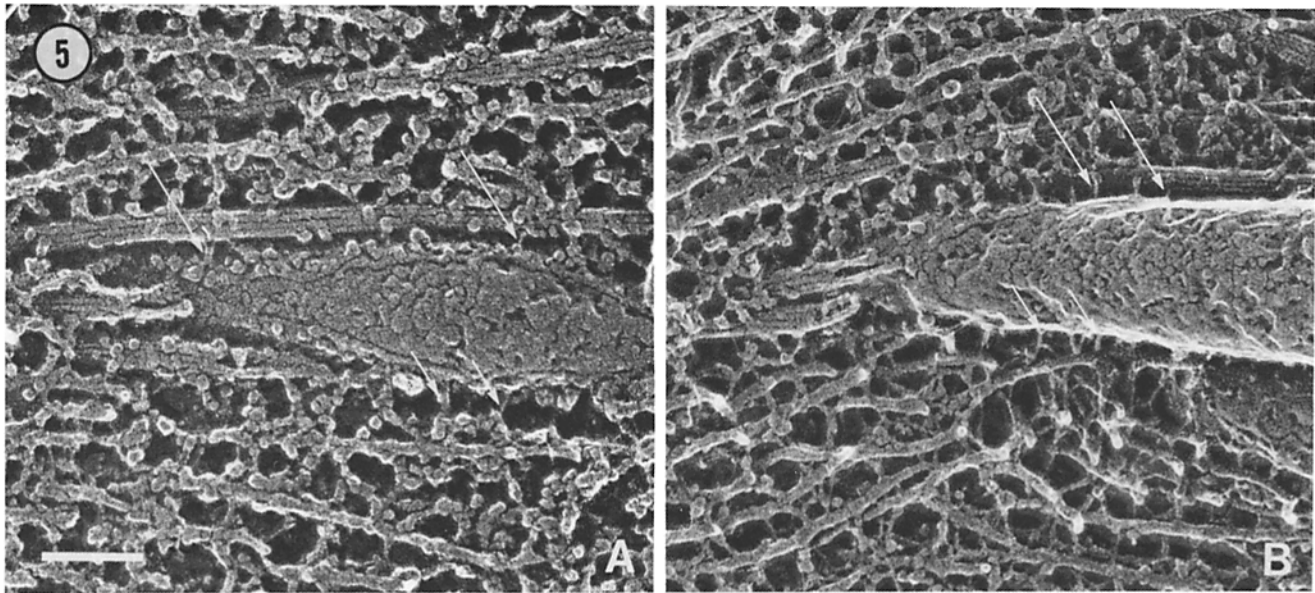
FIGURE 5 This micrograph shows the relationship between mitochondria, MT, and NF after various treatments. (A) Fresh frog axon. Cross-bridges (long arrows) between an MT and mitochondria and cross-linkers (short arrows) between a NF and mitochondria are displayed. (B) In a saponin-treated sample, in which most of the soluble proteins were removed, the same kind of cross-bridges and cross-linkers still exist. So, these structures are real. Bar, 0.1 μm. × 137,000.

the axon based upon a comparison of the images obtained with extracted tissue and with fresh unextracted axons.

NF and Cross-connections

NF ran longitudinally and parallel with each other and filled most of the axoplasm (Fig. 3). They were connected by numerous cross-linkers, (4-6 nm in diameter), mostly lying perpendicular to the long axis of the NF, thus giving them the appearance of ladders. The cross-linkers often branched and their length varied from ~20 to 50 nm depending on the distance between adjacent neurofilaments (Figs. 2 and 3). They were clearly observed after washing out most of soluble components from the axoplasm (Fig. 3). The distance between adjacent cross-linkers varied from 25 to 100 nm but was mostly from ~30 to 50 nm. The NF were linked to MT by fibrils 4-6 nm thin and 20-50 nm in length (Fig. 4B). The length and number of these fibrils were much more like those of the cross-linkers between NF than those of the cross-bridges between MT mentioned below (Fig. 4B).

MT and Cross-connections

MT were well preserved in the chemically and physically permeabilized axons by the use of taxol (39). They were identified as thick (25 nm in diameter), long, straight, tubular structures showing longitudinal protofilaments on the outside surface (Fig. 4). The fracture plane sometimes went through MT so that the inside was exposed to show parallel 4-nm stripes (Fig. 4A). The MT tended to exist as fascicles, and sometimes appeared to form channels (Fig. 1). MO, such as tubular membrane structures, vesicles of various sizes, and mitochondria, were often closely associated with them (Figs. 3, 5, 6, and 7). Very fine cross-bridges connected MT with each other. While they were also 4-6 nm in diameter, they tended to be less numerous and much shorter (<20 nm) than those between NF.

We could not find, however, any candidates for actin fila-

ments (8 nm in diameter displaying characteristic 5-nm periodicity) in the axoplasm of either unfixed whole axons or extracted axons (Figs. 1 and 3) except in the subaxolemmal space as described below.

MO and Cross-connections

Various types of MO were identified in the axoplasm including mitochondria, smooth endoplasmic reticulum (SER), vesicles of different sizes, and tubular membrane cisternae. Although the myelin and axolemma had numerous small pores after treatment with saponin, most of these organelles survived. They were covered by numerous bumps in the fresh axon. Mitochondria usually appeared rod-shaped and the fractured P face was characteristically studded by particles (Figs. 1, 5, and 6). They were very frequently associated with several MT (Figs. 1, 3, 5, 6). In fresh samples, as well as in extracted samples, we clearly recognized very short and thin cross-bridges (4-6 nm in diameter, <20 nm in length) between mitochondria and MT (Figs. 1, 3, 5, and 6). Cross-linkers between NF and mitochondria were also encountered (Figs. 5 and 6). They resembled those interconnecting the NF with each other with respect to their length and number (Figs. 3A and 6). SER was widely distributed in the axoplasm forming a long, branched, tubular cisternae system (data not shown). Cross-linkers were found between them and NF even after gentle homogenization of axons.

Vesicles of various sizes and tubular membrane structures were also common in the axoplasm. The former displayed ellipsoidal, dumbbell or spherical shapes (Figs. 1 and 7). The tubular membrane structures were sometimes difficult to discriminate from the SER, but they were much shorter and could be identified by their blind ends and lack of branching. These organelles tended to be localized quite near the MT bundles in the axoplasm. They could also be found in the subaxolemmal spaces. Wherever they were found, close associations with MT were common (Figs. 1 and 7). Frequently, cross-bridges between MT and these MO could be found in both fresh axons
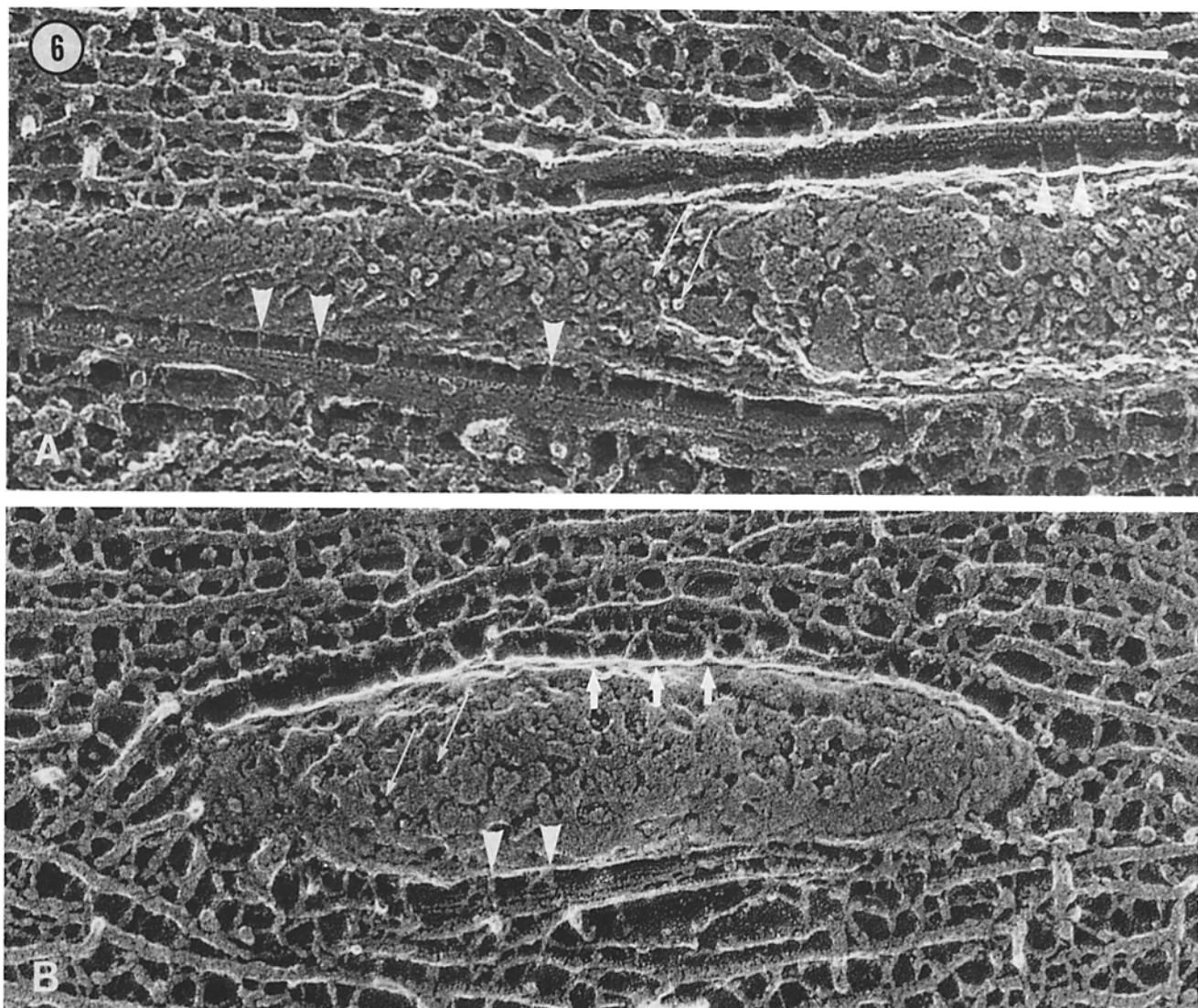
FIGURE 6 Relationship between mitochondria and cytoskeletal elements in saponin-treated axons. Long arrows indicate P face particles on the mitochondrial membrane. (A) The mitochondria is associated with MT at both sides. Short thin cross-bridges (arrowheads) are observed between MT and mitochondria. (B) The mitochondria is associated with NF and MT. Cross-linkers connect the mitochondria with NF (short arrows) and an MT (arrowheads). The cross-linkers between NF and mitochondria resemble those between NF. Bar, 0.1 μm. × 196,000.

and extracted axons (Figs. 1 and 7). Usually, most of them were very short and thin (4–6 nm in diameter, 10 nm in length) but sometimes longer prominent cross-bridges (20–30 nm in length) were also recognized in both fresh and extracted axons (Figs. 1, inset, and 7). The cross-linkers (mostly >20 nm) between MO and NF were another commonly encountered feature in both fresh and extracted axons (Figs. 1, 5, 6, and 7 B). They usually appeared similar in shape to the cross-linkers between NF.

Subaxolemmal Network of Filaments

The subaxolemmal space (~100 nm from the axolemma) was a differentiated region composed mainly of a dense network of long (50–150 nm) cross-links connecting MT and NF with the axolemma and subaxolemmal actinlike filaments (Figs. 8 B and 9). Occasionally, MT were enmeshed in network. The cytoplasmic surface of the axolemma was decorated with numerous 8–9-nm bumps, but the dense network of cross-links

made it difficult to analyze clearly the structure just inside the axolemma in the fresh axon (Fig. 8A). Physical rupture of a part of the axolemma by homogenization clearly demonstrated the existence of a network of filaments 8 nm or thinner just inside the axolemma (Figs. 8 B and 9). These filaments were distinct from the cross-links from NF and MT. Some appeared to be actin filaments (Fig. 8 B).

Localization of F-Actin in the Axon by NBD-Phillacidin Method

To further detect the localization of F-actin in the axon NBD-labeled phallacidin was used as a probe. The phallacidin is known to bind very specifically with both large and small F-actin but not with G-actin (1). In cross sections of axons, the fluorescence was detected mostly as two concentric circles or ellipses which corresponded to the positions of the axolemma (arrows in Fig. 10) and the Schwann cell cytoplasm (Fig. 10). The central parts of the axons were not stained (Fig. 10). This
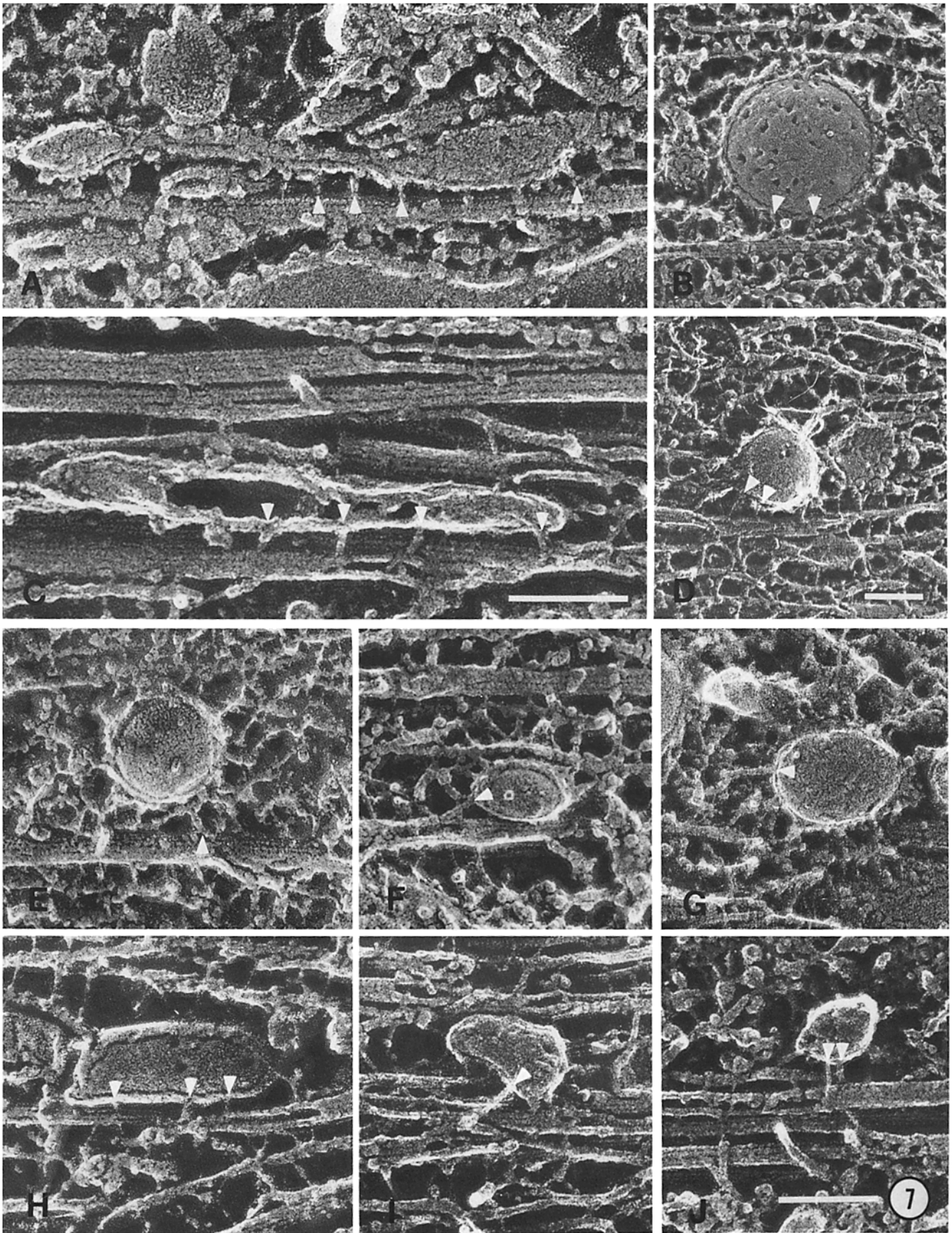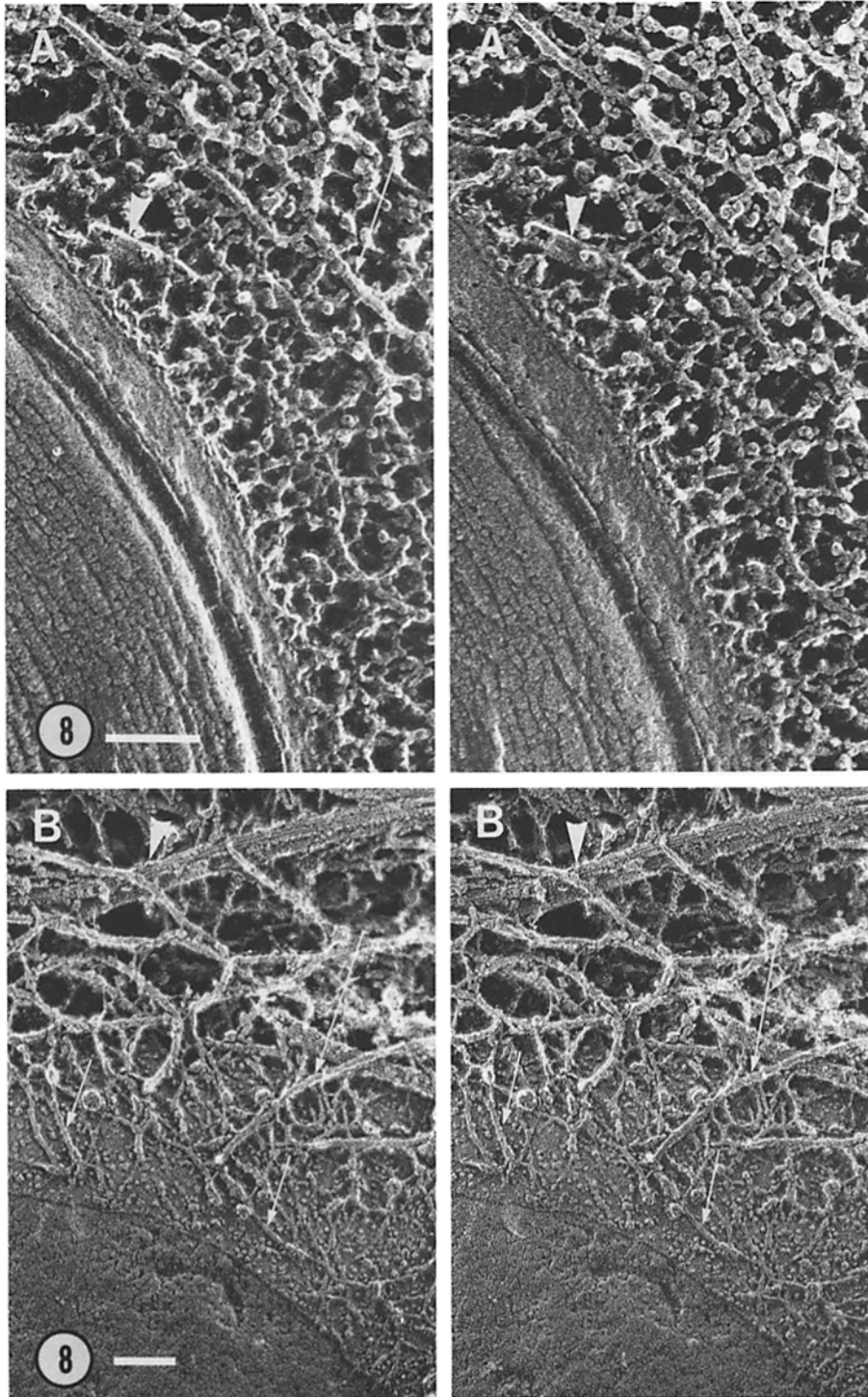
FIGURE 8 (*A*) This micrograph shows a stereo pair showing the subaxolemmal space in a fresh frog axon. The subaxolemmal space is filled with a network of cross-connections which connect NF (arrow) and MT (arrowhead) with the axolemma. MT tend to be very closely associated with the axolemma (arrowhead). Granular materials attached to filaments interfere with clear visualization of the inside of the axolemma. The myelin is observed at the lower left. Bar, 0.1 μm. × 130,000. (*B*) A stereo pair clearly displaying the inside of the *Torpedo* axolemma after gentle homogenization. The interior of the axolemma is decorated by numerous 8–9-nm protrusions. Also prominent is a network of 8-nm filaments (short arrows) thinner than the NF (long arrow) just inside the axolemma. Long cross-links (~100 nm) link MT and NF with the axolemma and the subaxolemmal network. Arrowheads: MT. Bar, 0.1 μm. × 83,000.

FIGURE 7 MO (possibly carried by the rapid transport system) in the fresh and saponin extracted frog axons. (*A*) A tubular membrane structure in a fresh frog axon. It is linked with an MT by fine cross-bridges (arrowheads). (*B*, *D*, and *E*) Spherical membrane organelles closely associated with a MT in fresh frog axon (*B* and *E*) and saponin-treated axon (*D*). Cross-bridges are indicated by arrowheads. (*C*) A tubular membrane structure in a saponin-treated frog axon. It is closely associated with MT linked by fine short cross-bridges. (*F* and *G*) Spherical MO in the fresh frog axon. It is linked with a MT by a prominent long cross-bridge (arrowhead) (*F*). The same kind of prominent long filament (arrowhead) disappears in the granular material (*G*). (*H*, *I*, and *J*) Cross-bridges (arrowheads) between MT and MO in saponin-treated frog axons. Prominent long cross-bridges are found in *I* and *J*. Bar, 0.1 μm. (*A* and *C*) × 226,000. (*B* and *D*) × 111,000. (*E*, *F*, *G*, *H*, *I*, and *J*) × 183,000.
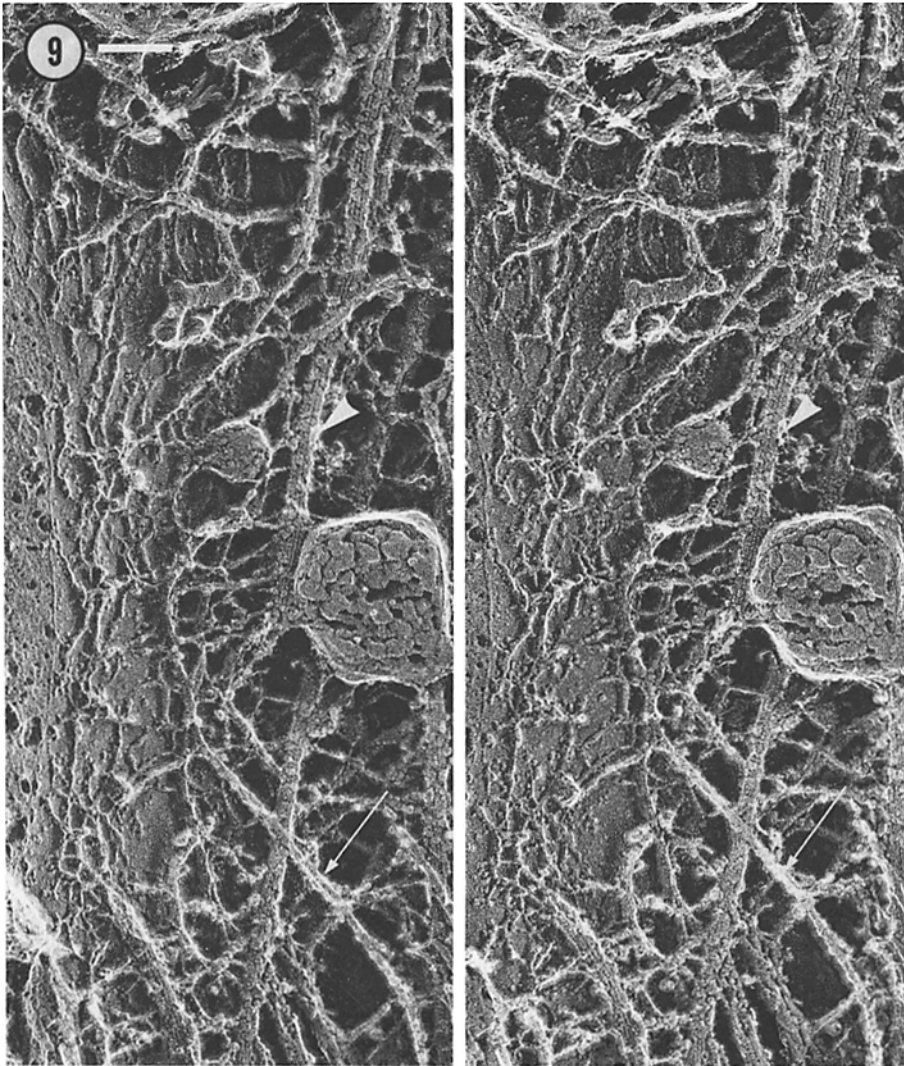
FIGURE 9 A stereo pair of the inside of the axolemma of physically broken *Torpedo* axon. Thin longer cross-links (>100 nm) between MT (arrowhead) and axolemma are prominent. A network of thin filaments occurs just inside the axolemma. Long arrow: NF. Bar, 0.1 μm. × 96,000.
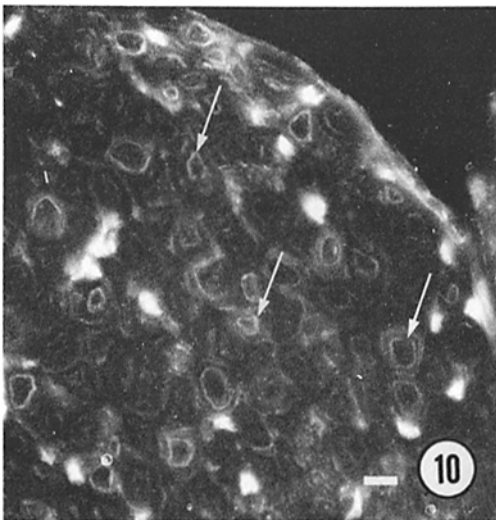

FIGURE 10 Fluorescence micrograph of a cross section of the frog sciatic nerve stained with NBD-phallacidin to detect the localization of F actin. Fluorescence is detected as two layers of concentric circles or ellipses. Inner circle or ellipse (arrows) and outer one correspond with the position of the axolemma and Schwann cell cytoplasm respectively. The bright spots seem to be vascular endothelial cells. Bar, 10 nm. × 430.

result supports the localization of actinlike filaments just underneath the axolemma as demonstrated by the deep-etching method.

DISCUSSION

Methodological Problems for Observation of Cross-connections

The main purposes of this study were (a) to investigate whether the elaborate cross-connections observed in the axoplasm are real or artifactual; (b) if real, to examine their relationship to MO; and (c) to determine whether cross-connections can be categorized structurally into different groups related to possible functions. Salt or soluble proteins in the fresh axons could easily imitate tiny structures like cross-connections by precipitation or condensation in the process of deep-etching. To investigate whether salt could produce any artifacts, fixed tissues (containing considerable salt) were directly quick-frozen and compared with the fixed tissues washed extensively with distilled water before freezing and with fresh tissues. There were no detectable differences between them. Basically, the morphology of fixed tissue appeared to be similar to that of the fresh axon except that the fixation obscured the surface substructure of the MT. Furthermore, we could not

find any possible artifactual filamentous system at the extracellular spaces of the fresh samples. This is important in view of the recent finding of Miller and Lassignal (32) that salt could produce artifactual filamentous networks after freeze-drying of the quick-frozen samples. The difference between the freeze-fracture–deep-etching method and the simple freeze-drying method should be stressed. In the former the sample is first freeze-fractured and then etched for <5 min at −95°C so that only a shallow layer of ice in the cytoplasm is etched, but in the latter the sample is etched for a much longer time (~1 h at −95°C) so that a thick layer of ice on the samples is totally etched. These differences apparently account for the reduction of artifact in the freeze-fracture–deep-etching method.

Next, the possibility that soluble proteins condense artifactually to form the cross-connections in the fresh and fixed axons was studied. In both fresh and fixed whole axons, the cross-connections appeared to be the same. Occasionally, knobby materials were attached to them as well as the NF and MT. Soluble proteins from the axoplasm were removed by two methods. One method was to incubate the axon with saponin-containing medium (21, 22). Saponin perforated the axolemma and myelin. Consequently, most of soluble proteins seemed to leak out of the axoplasm and the cross-connections appeared to be smooth in contour, but with most of the attached knobby materials gone. The other method of washing out soluble proteins was to homogenize the nerve gently. This process broke a part of the axolemma. Even after this kind of physical distortion, the cross-connections often remained and the cytoskeletal components retained their relatively intact spatial relationships in the axon. The cross-connections that survived this treatment resembled those in the saponin-treated axons. Taxol (39) is necessary to stabilize the MT in these extraction experiments, probably because the tubulin pool in the axoplasm has leaked out. It can be speculated that the granular materials removed by physical or chemical extraction could be soluble components, perhaps proteins, and that some of these soluble proteins either become attached or were originally attached to the cross-connections, NF and MT in the axoplasm. My results establish that the cross-connections between NF, microfilaments, MT, and MO are real structures in the living axon and are not artifacts produced by precipitation of either salt or soluble proteins during fixation or quick-freeze–deep-etching. This study also shows that, in the fresh or fixed whole axon, cytoskeletal elements were less obscured by granular substances than in the other cell system previously reported such as intestinal epithelial cells (21). This may be because the local concentration of soluble proteins is different in various kinds of cells and in distinct parts of the same cells.

Concerning the structure inside the axolemma, saponin treatment allowed clear visualization of cross-connections and cytoskeletal filaments without physical distortion, even though the axolemma was affected. On the other hand, physical rupture of a part of the axolemma by gentle homogenization allowed good preservation of the structures just inside the axolemma. This method clearly revealed the filamentous networks inside the membrane. The same procedure has also demonstrated the relationship between the membrane and cytoskeletal elements in intestinal brush borders and neuromuscular junctions (21, 22). Thus, combining all the data from fresh whole axons and chemically and physically extracted axons, it appears that the real three-dimensional architecture of the axon is dependent on the interaction between filaments and MO and the elaborate cross-connection system.

The images of cross-linkers between NF revealed by this method are consistent with those observed by high voltage electron microscopy of thick sections, but different from images obtained by embedding medium-free sections (PEG method) in a previous study (10).

Cross-connection System between NF, MT, and MO

The data established the following in unfixed axons. Most of the axoplasm was filled with longitudinally oriented neurofilaments (NF). Within the axoplasm, two specialized regions were seen: (a) MT-rich regions, which often contained fascicles of MT; (b) a subaxolemmal space, which was composed mainly of a network of long cross-links from MT and NF. MO, e.g., vesicles of various sizes (probably multivesicular bodies, lysosomes, and small vesicles), as well as tubular membrane structures (45) and mitochondria, were located mainly in the a and b regions. On the basis of their location, population and length, the cross-connections could be generally categorized into three different groups which may reflect the distinct function and nature of the cross-connections. (i) NF-associated-cross-linkers which connected NF to NF, NF to MT, NF to MO; (ii) MT-associated-cross-bridges which linked MT to MO and MT to MT; and (iii) long cross-links (from ~50 to 150 nm in length) in the subaxolemmal space. The cross-linkers of i were numerous and existed extensively throughout the axoplasm and were from ~20 to 50 nm in length. Cross-bridges in ii (from ~10 to 20 nm in length) were mostly shorter than in i and less frequent. A few longer cross-bridges were observed between MT and MO, raising the possibility that they could be elastic.

Structurally, the cross-linkers associated with NF are unique to nerve cells. Such cross-linkers have not been found by the quick-freeze–deep-etch method in the intermediate filaments composed of vimentin (55 to 58 kdaltons) (19) in the fibroblasts or tonofilaments composed of prekeratin associated with desmosomes in epithelial cells (21, 23). Intermediate filaments in glial cells constituted from glial fibrillary acidic protein (55 kdalton) (36, 38) or vimentin do not show cross-linking structures either (unpublished data). These cross-linkers could not be formed by F-actin or myosin, both of which are reported to be axonal constituents (5, 26, 50) and which could theoretically form filamentous structures. They were too thin to be F-actin (8–9 nm in diameter) and did not display its characteristic morphology (19), and they were too short to be myosin (the length of the monomeric myosin is ~150 nm [35]). On the other hand, two recent immunocytochemical studies using antibodies against three triplet proteins (200, 145, 73, or 68 kdaltons) have reported that the 68 or 73-kdalton protein seems to form the backbone of the filaments, but the 200-kdalton protein is periodically arranged (repeated ~100 nm) in a more peripheral position (40, 49). Thus, Willard and Simon (49) and Sharp et al. (40) have suggested that the 200-kdalton protein could form cross-linkerlike structures. Although they could not preserve the cross-linkers between NF in their preparation, and although the actual distance between most of adjacent cross-linkers was <100 nm, their speculation fits well with the ladderlike structural characteristics of the cross-linkers observed in this study. The quick-freezing method combined with immunocytochemistry as done in the brush borders (23) should provide the final answer to this question. Since the cross-linkers are extensively developed throughout the axoplasm, they could constitute a labile and elastic framework. In addition, by connecting NF with each other and with MT as a unit, they may have consequences on the slow transport of the NF and MT (2, 24, 33).

The cross-bridges in (*ii*) associated with MT could be composed of MT-associated proteins (MAPs). In fact, it has been suggested that MAPs could form cross-bridgelike structures (19) and could connect actin filaments with MT as multiple bonds of fixed length (15). It is also possible that the same cross-bridges contribute to form some of the cross-linkers between MT and NF. A second possibility is that the cross-bridges may have some ATPase activity to generate a force for MO sliding on the MT. But the cross-bridges are small (mostly 4 ~ 6 nm in diameter ~10 nm in length) and strucurally different compared with well-characterized dynein ATPase associating with MT (18). The dynein was shown to be composed of an eliptical head 16 × 19 nm and a 12-nm long slender stalk and arm by the quick-freeze–deep-etch technique (18). Moreover, one report has demonstrated that MAPs do not have ATPase activity (34). Thus, biochemical analyses are necessary to elucidate the function of the cross-bridges observed here.

The cross-links in *iii* in the subaxolemmal space are long (sometimes >100 nm) and connect the subaxolemmal actinlike filamentous network and axolemma with NF and MT. A similar kind of cross-link has been found to connect intermediate filaments with actin filaments in the brush border of intestinal epithelial cells (23). One of the candidates for cross-links could be fodrin, which interacts with actin and has been specifically localized beneath the axolemma (28). The molecular weight of fodrin (250,000, 240,000) is very close to myosin which forms small bipolar filaments about 175 nm in length (35). MAPs could be the other candidate. We will be able to learn more about the nature of these cross-connections by the present method combined with immunocytochemistry as done in another system (23).

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