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Axonal Microtubules in Squid Giant Axons Observed under an Optical Microscope and Their Structural Relation to the Membrane Excitability

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Axonal microtubules in squid giant axons were observed under an optical microscope. Physiological conditions of axons under microscope were controlled by perfusing intracellularly with artificial solutions and by clamping membrane voltage to measure membrane currents. We found that axonal microtubules ran nearly parallel to longitudinal direction of axons, and that they were mainly distributed at locations very close to the axolemma through 20 μm apart from it for the axon with full excitability whether the axon was intracellularly perfused or intact. The density of these peripheral microtubules was reduced in the axon whose excitability was partially destroyed by perfusing with a solution unfavorable for the excitability. The degree of reduction of the microtubule density was well correlated to that of suppression of the excitability, as far as we had ever tested. These observations suggest the importance of axonal microtubules on the maintenance and generation of membrane excitability in squid giant axons.

Axonal microtubules appear to play an important role in maintenance and generation of membrane excitability in squid giant axons. Evidences that axonal microtubules are necessary for the excitability have been obtained by observing physiological responses of the axon when it is exposed to conditions favorable or unfavorable for microtubule assembly. Internal application of reagents suppressing microtubule assembly to the axon was found to bring about a decrease in the maximum size of Na conductance, shift of the voltage dependence of the normalized peak Na conductance in the positive direction along voltage axis and a decrease in the size of Na actvation gating current (Matsumoto *et al.*, 1984b). Further, it was found that the reduced Na current was restored by perfusing axons intracellularly with a solution containing microtubule proteins consisted of fully tyrosinated tubulin and axolinin under the conditions supporting microtubule assembly (Matsumoto *et al.*, 1984a). It was also found that the gating current had two components; the one (colchicine-sensitive part) disappears when the axon is intracellularly perfused with a solution containing colchicine, while the other (colchicine-insensitive part) remains under the colchicine treatment. It was found that the colchicine-sensitive part of the gating current was quantitatively correlated to the actvation of Na conductance (Matsumoto and Ichikawa, 1985).

Recent progress in optical image processing techniques has made it possible to observe axonal microtubules in squid giant axons. We will get more direct information of physiological role of axonal microtubules when we apply these techniques to the axons whose physiological conditions are electrically controlled under the optical microscope. Here we report a

structural correlation of axonal microtubules to the membrane excitability with the microscopic observation technique under physiological control.

Materials and Methods

Materials

Giant axons of squid (*Doryteuthis bleekeri*) were used. Squid were collected in Sugami bay, transported to the Electrotechnical Laboratory and maintained in a closed-system laboratory tank (Matsumoto, 1976; Matsumoto and Shimada, 1980).

Axon diameters were between 300 and 600 µm. The majority of the adherent tissues surrounding the axon was removed under a dissecting microscope.

Methods

We constructed an experimental system which made it passible to observe axonal microtubules in intracellularly perfused axons under an optical microscope. Physiological conditions were controlled with the voltage clamp method. Schematic diagram of the experimental system is illustrated in Fig.1. The system consisted of an optical microscope (New VANOX AH2, Olympus Co.), a TV camera (Hamamatsu Photonics Co.), an image processor (Photonic-microscope-system C1966, Hamamatsu Photonics Co.) and a physiological experiment system which had been described in detail in the other paper (Matsumoto *et al.*, 1984a).

Nomarski differential interference method was adopted to observe axonal microtubules, where X40-dry or X100-oil splan objective lens were used. The image processor has the following characteristics; 640 × 480 pixels in resolution and 8 bit gray scale in dynamic range. The observed image data were recorded with VTR (U-matic STD. VO-5800, SONY Co.). A personal computer system was added to the Hamamatsu Phonics image processor to process the digital image data further.

The voltage clamp method with series

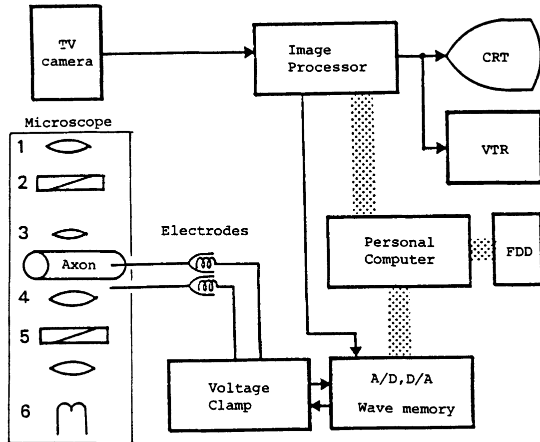

Fig.1: Schematic diagram of the experimental system. The optical microscope is basically composed of 6 units; expansion lens (1), Nomarski prisms (2 & 5), objective lens (3), condenser lens (4) and tungsten lamp.

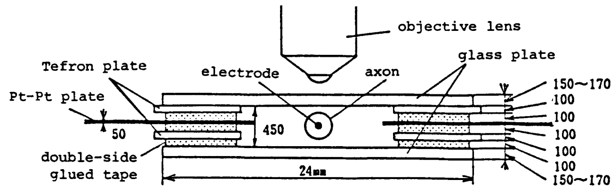

Fig.2: Cross-sectional view of the chamber in which the squid giant axon is put. External solution surrounding the axon in the chamber is perfused not to disturb microscopic observation of axonal microtubules.

resistance compensation was used. A command pulse was prepared with a personal computer system for voltage-clamping. Membrane currents were amplified, converted into digital signals with an A/D converter (12 bit, 2 μsec) and stored into a wave memory (16 KW) under the control of the personal computer.

Chamber

A chamber with a bottom glass wall of 0.15 - 0.17 mm in thickness was used (Fig.2) for both optical observation and electrophysiological experiments. It was consisted of a glass plate at the bottom (24 × 24 mm in size, 0.15 - 0.17 mm in thickness), a Tefron plate (0.1 mm in thickness) for spacing (see Fig.2), a glass plate to cover the chamber (the same one as the bottom plate) and a platinized platinum plate for external guard electrode. These two plates were pasted with double-side glued tape (3M Co.), as seen in Fig.2. The chamber could contain 0.06 ml external solution. The temperature of the axon was kept constant at any temperature from 16 through 20°C by circulating temperature-controlled water around the chamber.

Intracellular Perfusion

The axon was intracellularly perfused with the modified roller method

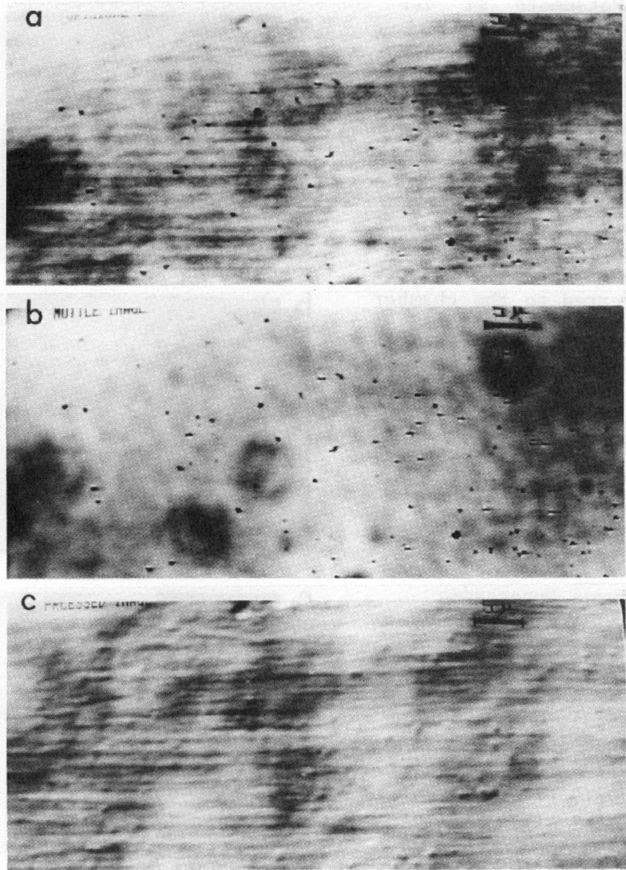

Fig.3: An example to illustrate how to process the image of axonal microtubules inside squid giant axon. *a*, original image. *b*, mottle image. *c*, processed image (see the text). Objective lens × 40 and expansion lens × 4 are used.

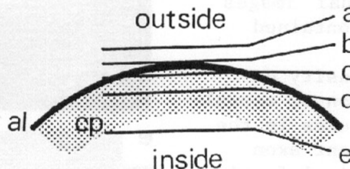

Fig.4: Schematic drawing of focal plane for microscopic observation. *a*, ouside of axon. *b*, on the axolemma (0 μm). *c*, inside axon 2 μm apart from the axolemma (inside 2 μm). *d*, inside 6 μm. *e*, inside 12 μm. In the figure *al* and *cp* stand for axolemma and cytoplasm, respectively.

(Matsumoto *et al.* 1984a). After the axon was put on transparent rubber plate (silicon rubber plate), a central part of axoplasm was gently extruded using a rubber-coated roller while the axon was kept straight. The axon was then transferred to the chamber filled with ASW (400 mM NaCl, 44 mM $CaCl_2$, 10 mM KCl, 10 mM Tris.-HCl, pH 8.1) and was initially perfused with 355 KF-SIS (355 mM KF, 25 mM K HEPES, 4% glycerol, pH 7.3).

Image Processing

We adopeted an AVEC method for digital image processing (Allen and Allen, 1983). The general procedure was done with the following way: First the mottle image (the out-of-focus image) was freezed in a frame memory. Then the original image was taken for the axonal microtubules after averaged for 4 to 8 frames. Both mottle and original images commonly contained

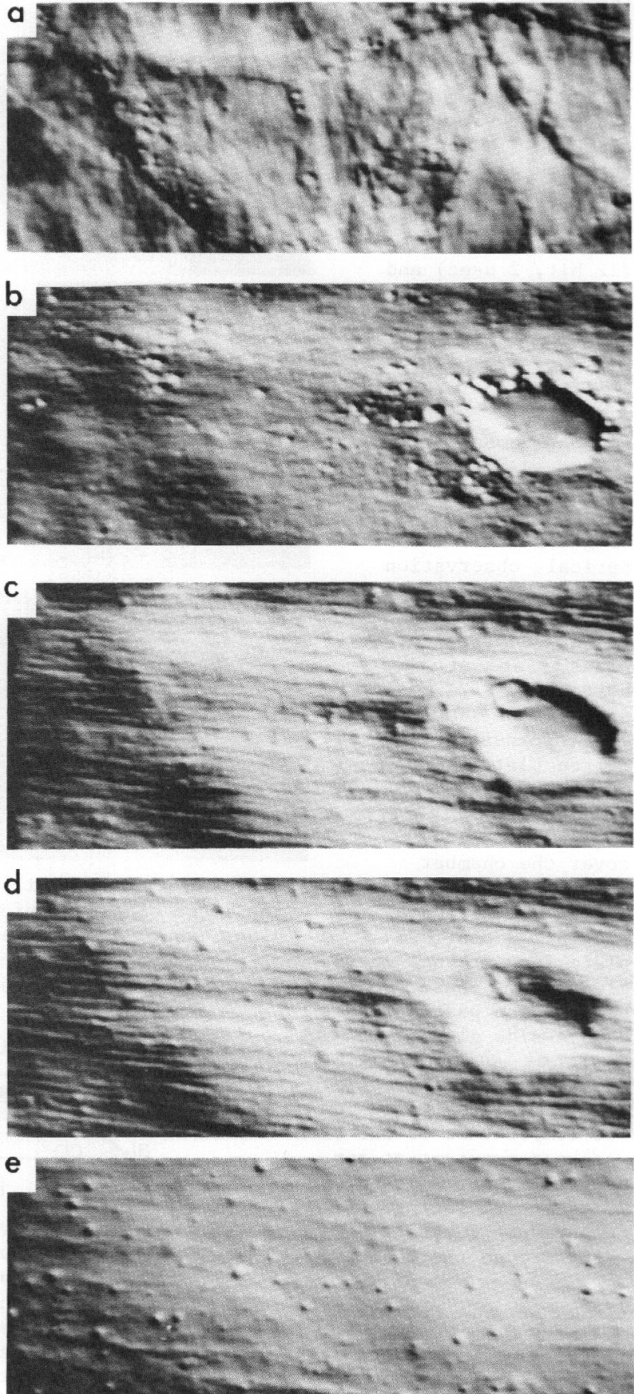

Fig.5: Density destribution of axonal microtubules in the squid giant axon when optical focal plane is changed; *a* out side of axon, *b* on the axolemma, *c* inside 2 μm, *d* inside 6 μm and *e* inside 12 μm (see Fig.4).

almost the same dust shadows and image inhomogeneity due to unbalanced illumination and/or large size of tissues. As such, the original image after mottle subtraction was greatly improved after subtracting the mottle image from each averaged images. The improvements of image contrast, image resolution and uni-focal image were clearly recognized, as shown in Fig.3.

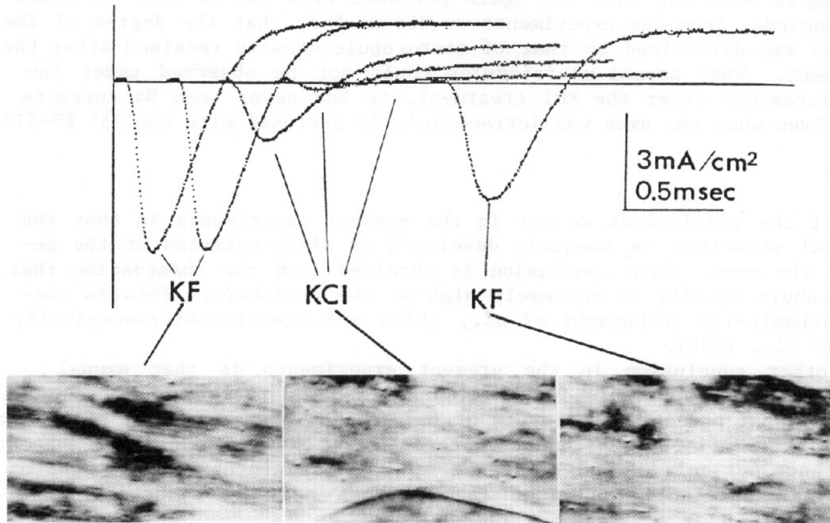

Fig.6: Effect of internal KCl-HEPES solution both on Na & K ionic currents (upper curves) and on axonal microtubule densties under an optical microscope (lower plates). Both electrophysiological experiments and the microscopic observation are done for the same axon at the same time (see the text for experimental procedures). Abscissa in the upper figure represents time after intracellular perfusion is started. The axon is initially perfused with KF solution for control (the left two records), then with KCl-HEPES to block Na & K ionic currents (the central three records) and finally with KF solution again to test whether or not the ionic currents are restored (the extremely right record). In this example the currents are partially restored.

Results

Distribution of axonal microtubules was observed under an optical microscope as a function of distance from the axolemma to the interior of the axon. This was done by changing focal plane (Fig.4). One of the examples for these experiments is shown in Fig.5 : In Record *a* we see connective tissues surrounding Schwann cells. We see many small dimples separated 3 to 5 μm from each other in Record *b*. Record *b* was taken from the location very close to the axolemma. Records *c*, *d* and *e* were taken from the separate locations inside the axon. We found the maximam density of microtubules around at locations 2 μm distant from the axolemma. The number density was rapidly reduced as we went into the interior of the axon. As shown in Fig.5e, at the location 12 μm from the axolemma, we can see only small number of microtubules and of particles associated with microtubules.

Next we studied structural changes of axonal microtubules when the

electrical excitability was modified by perfusing the axon intracellularly with 355 KF-SIS or 355 KCl-HEPES, where the 355 KCl solution contained 355 mM KCl, 25 mM K-HEPES (pH 7.3) and 4% glycerol. Perfusion with the 355 KCl solution suppressed Na currents rapidly. At the same time we found the density of microtubules was reduced. When we could see some axonal microtubules remained after the KCl treatment, we found Na currents were restored to some degree when the axon was again perfused with 355 KF-SIS. We could tend to conclude, from our experiments we did so far, that the degree of the restoration was determined by that of microtubule density remained after the KCl treatment. When axonal microtubules could not be observed under the optical microscope after the KCl treatment, we had never seen Na currents recovered even when the axon was intracellularly perfused with the 355 KF-SIS.

Discussion

One of the conclusions we got in the present experiments is that the cytoskeletal structure is unequely developed or differentiated at the periphery of the axon. This conclusion is obtained from the observation that the microtubule density is extremely high at the periphery. This is confirmed biochemically (Kobayashi *et al.*, 1986) and electron-microscopically (Tsukita *et al.*, 1986).

The other conclusion in the present experiments is that axonal cytoskeleton is indispensable for maintenance and generation of Na channels. Particularly, the present experiments of suppression of Na currents after the KCl treatment and of the subsequent restoration by the KF application suggest the importance not only of the existence of axonal microtubules but also of some specific interaction between the axolemma and the cytoskeletal structure.

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