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Are Axoplasmic Microtubules Necessary for Membrane Excitation?

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Summary. The excitability of the squid giant axon was studied as a function of transmembrane hydrostatic pressure differences, the latter being altered by the technique of intracellular perfusion. When a KF solution was used as the internal medium, a pressure difference of about 15 cm water had very little effect on either the membrane potential or excitability. However, within a few minutes after introducing either a KCl-containing, a KBr-containing, or a colchicine-containing solution as the internal medium, with the same pressure difference across the membrane, the axon excitability was suppressed. In these cases, removal of the pressure difference restored the excitability, indicating that the structure of membrane was not irreversibly damaged. Electron-microscopic observations of these axons revealed that the perfusion with a KF solution or colchicine-containing solution preserves the submembranous cytoskeletal layer, whereas perfusion with a KCl or KBr solution dissolves it. These results suggest that the submembranous cytoskeletons including microtubules provide an important mechanical support to the excitable membrane but are not essential elements in channel activities.

Key Words squid · axon · Na channel · cytoskeleton · colchicine · microtubules

Introduction

Microtubules, intermediate filaments and microfilaments form a three-dimensional array in the cytoplasm of various eukaryotic cells. In nerve cells, these filamentous proteins are closely related to the axoplasmic transport (Dahlström, 1968; Karlsson & Sjöstrand, 1969; Kreutzberg, 1969; Lasek & Hoffman, 1976) and the development of the growth cone (Seeds et al., 1970; Yamada et al., 1970; 1971; Wessels et al., 1971). However, the relevance of filamentous proteins, or cytoskeletal proteins, to the nerve excitability has been a matter of controversy. Some investigators hypothesized that reducing the number of microtubules with general anesthetics (Allison & Nunn, 1968), colchicine (Rodriguez-Echandia et al., 1968), or other antimitotic drugs (Mitolo-Chieppa, 1977) suppressed the ability of nerve cells to produce action potentials. Others reported that colchicine has no effect on impulse conduction in nerve fibers (Fernandez et al., 1971; Hinkley & Green, 1971; Albuquerque et al., 1972; Jackson & Diamond, 1977; Schafter & Reagan, 1981) or it even augments the ability to produce action potentials in normally inexcitable neurons (Pitman et al., 1972; Pitman, 1975). Recent studies revealed a wide variety of effects of colchicine on ion-channel activities. Colchicine blocks a type of the K channel in crayfish axons (Terakawa & Watanabe, 1976), the Ca channel in cultured guinea pig neurons (Fukuda et al., 1981), the Na channel in squid giant axons (Matsumoto & Sakai, 1979*a*; Matsumoto et al., 1980, 1984*a*,*b*; Chang, 1983; Landowne et al., 1983), or it blocks the Ca, Na and Cl channels in *Aplysia* neurons (Baux et al., 1981). The diversity of these findings suggest that cytoskeletal proteins influence the activity of ion channels in an indirect way via some unknown factors.

In this study, effects of chaotropic anions and colchicine on the squid axon membrane were examined with a particular emphasis on the correlation between electrophysiological and morphological changes. The results were highly dependent on the presence or absence of mechanical stresses on the membrane. Conventionally, the intracellular perfusion is performed with the intracellular pressure set at 10 to 25 cm H_2O. Previously, little attention has been paid to this pressure. Yet, it is this pressure that causes the mechanical stress. The stress reversibly suppresses the excitability when the membrane lacks cytoskeletal protection. It will be shown that axoplasmic fibrous structures including microtubules provide the membrane with mechanical strength and rigidity, but these structures are not essentially involved in the molecular machinery of ionic channels.

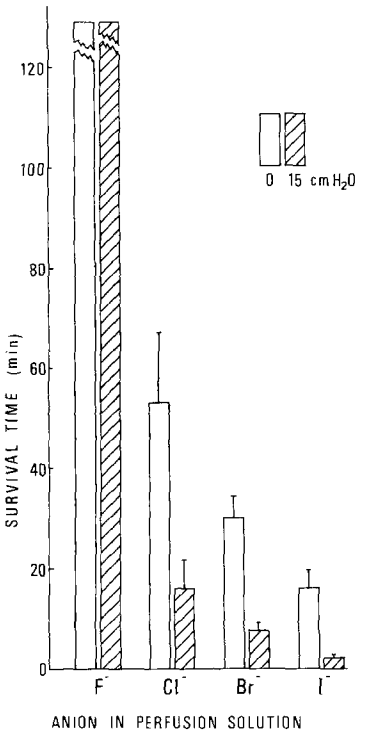


Fig. 1. Effect of intracellular pressure on the survival time of the axons perfused with solutions containing various halogen ions. Each mean (column) and half of standard error (vertical bar) were obtained from 7 axons perfused with a 360 mM KCl, KBr, or KI solution and from 3 axons perfused with a 360 mM KF solution. Open columns: the intracellular pressure was set at 0 cm H_2O. Hatched columns: the pressure was set at 15 cm H_2O. The survival time of axons perfused with the KF solution was more than 120 min irrespective of the pressure

Materials and Methods

Internal Perfusion and Electrophysiological Measurements

The giant axon excised from the squid (*Loligo pealei* and *Dorytheusis bleekeri*) was mounted horizontally in a Lucite® chamber containing seawater, and intracellularly perfused by the double cannulation method (Tasaki et al., 1965). The inlet pipette was 130 μm in inside diameter and 22 mm in length. The outlet pipette was 240 μm in inside diameter and 26 mm in length. The diameter of axons used ranged from 400 to 650 μm. The length of perfusion zone was 16 to 20 mm. The intracellular pressure was applied by a reservoir connected to the inlet pipette through a polyethylene tube. The height of reservoir was adjusted by a manipulator with a 0.1 mm accuracy. The level of the axon served as an origin for the pressure measurement. When both resting and action potentials were measured, an electrode consisting of a glass pipette (150 μm in outer diameter), Ag-AgCl wire, and 0.6 M KCl-agar was used. This electrode was inserted into the axon through the outlet pipette. Usually, the internal solution was allowed to flow through the outlet pipette.

Intracellular pressure was determined as follows: A glass capillary connected to a syringe with a polyethylene tube was inserted into the axon through the outlet pipette. This syringe supplied a colored solution to the tip of the capillary. The height of the syringe was adjusted so that the colored solution did not move at the tip of the capillary. The height was measured from the level of the axon, and was taken as the intracellular pressure for the case where the intracellular solution was allowed to flow. The intracellular pressure thus measured varied linearly with the pressure applied by the reservoir (proportionality constant: 0.4 to 0.5). The pressure probe was removed prior to the electrical measurements.

When only action potentials were measured, a Pt-wire electrode (50 μm in diameter) was used internally. In this case, a silicone-rubber tube was connected to the outlet pipette and the Pt-wire electrode was inserted into the axon interior through a small hole made in this tube. The flow of perfusion solution could be blocked by clamping the silicone-rubber tube. Using this method, the hydrostatic pressure inside the axon was made uniform, and the value of the pressure could be measured directly from the height of the reservoir. All electrophysiological observations were performed at room temperature (19 to 23°C).

Solutions

In evaluating the effects of chaotropic anions, a solution containing 4% glycerol and 400 mM K salts was used intracellularly. Ninety per cent of the anion was either F^-, Cl^-, Br^- or I^-, while 10% was phosphate added as a buffer of pH 7.3 ± 0.1. When the effects of colchicine were examined, a solution containing 8% glycerol, 200 mM K^+, 180 mM F^- and phosphates was used as the internal medium. Colchicine (Sigma, St. Louis, Mo.) was added directly to this medium. The external medium was artificial seawater containing 450 mM NaCl, 100 mM $CaCl_2$ and 1 mM Tris-HCl (pH 8.1). At the initial stage of perfusion, a solution containing 0.05 mg/ml Protease VII (P5255, Sigma) was used for about 1 min.

Electron Microscopy

The axon was fixed for 2 hr in the Lucite chamber with 2.5% glutaraldehyde added to the natural seawater. The action potential was suppressed by the fixative within 2 min. Next, the axon, with Schwann cells attached, was incised longitudinally at the perfusion zone for a length of about 1 cm so as to expose its interior. Then the axon was removed from the chamber and rinsed in a 200 mM Na-cacodylate buffer at pH 7.3 for 15 min. Postfixation was performed with 1% OsO_4 in a 200 mM Na-cacodylate buffer for 1 hr. After rinsing with distilled water, the axon was dehydrated in a graded series of ethanol-water mixtures (50, 75, 90, 95%, and twice of 100%). Ethanol was then replaced with iso-amylacetate. The specimen was critical-point dried with CO_2, sputter-coated with gold and observed under a scanning electron microscope (S-800, Hitachi) at an acceleration voltage of 25 kV. For transmission electron microscopy (200CX, JEOL), the ethanol-dehydrated specimen was embedded in Spurr's resin (Spurr, 1969) and cut into ultra-thin sections. These were stained with 1% uranyl acetate and 1.5% lead citrate. All procedures were performed at room temperature (19 to 23°C).

SDS-Polyacrylamide Gel Electrophoresis

Proteins collected in the perfusate were solubilized by the addition of 0.1% sodium dodecyl sulfate (SDS) and by heating for 3 min in boiling water. The solubilized sample was dialyzed against

distilled water and then freeze-dried. The sample was further dissolved in a buffer consisting of 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS, and 62.5 mM Tris-HCl (pH 6.8). This solution was heated for 3 min and centrifuged at 10,000 × *g* for 5 min. The clear supernatant was placed in a well of 10% polyacrylamide gel (8 × 8 cm, 1 mm thick) and subjected to electrophoresis (Laemmli, 1970). Next, the gel was fixed with a 10% acetic acid-45% methanol solution and silver-stained (Oakley et al., 1980). Molecular weight markers used were: myosin (200 kD), β-galactosidase (116 kD), phosphorylase *b* (94 kD), bovine serum albumin (68 kD), α- and β-tubulins (55, 53 kD), ovalbumin (45 kD), and carbonic anhydrase (29 kD).

Results

Effects of the Intracellular Pressure on the Survival Time of Perfused Axons

In a series of experiments, the survival time, i.e., the period from the onset of internal perfusion to the loss of excitability, was examined; the intracellular pressure was maintained at either 0 or 15 cm H_2O and the anion in the perfusion solution was varied from F^- through Cl^-, Br^- and I^-. The intracellular solution was allowed to flow for an initial 2 min to exchange the internal medium, then it was blocked at the tube connected to the outlet pipette. Once the pressure was set, it was left unaltered. Loss of excitability was ascertained when the amplitude of the action potential became smaller than 40 mV. The results are shown in Fig. 1. The order of the ability of various anions to maintain excitability was the same as that reported by Tasaki et al. (1965). However, the survival time measured with the same anion was greatly affected by the pressure: the survival time at 0 cm H_2O was more than twice that at 15 cm H_2O. This was true for all axons perfused with Cl^-, Br^-, and I^--containing solutions.

Axons Perfused with KF Solutions

In the case of axons perfused with a solution containing KF as the major salt, markedly high pressures (more than 40 cm H_2O) had to be applied intracellularly to induce depolarization of the membrane and to block excitability. It was extremely difficult to induce a large and reversible effect of intracellular pressure on axons perfused with a KF-containing solution. However, small changes in ionic currents could be demonstrated by the voltage-clamp method (Fig. 2). Normally, in the axon perfused with a 180 mM KF solution, the maximum inward current was 2 to 3 mA/cm^2 when the intracellular pressure was 13 cm H_2O. The inward current was increased by 5 to 15% when the intracellular pressure was reduced to 0 cm H_2O. The outward

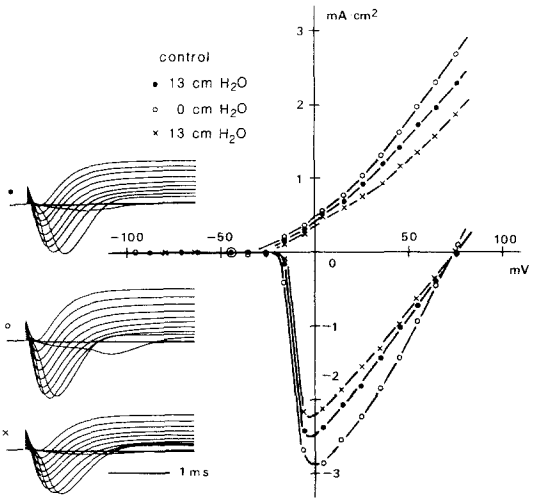


Fig. 2. Pressure sensitivity of the 180 mM KF-perfused axon. Families of membrane currents (left) were obtained 15 min after varying the intracellular pressure to 13, 0 and 13 cm H_2O again by the voltage-clamp method. Commanding pulses of 10-mV steps were applied from the holding potential of −45 mV. The *I–V* curves (right) were obtained from these membrane currents

current also increased slightly. Raising the pressure reduced both currents significantly. The membrane currents quickly changed in 5 to 10 min and then slowly over a period of a few hours. The reversal potential was only slightly affected by the intracellular pressure, indicating that the amplitude of the action potential was rather insensitive to the pressure.

Scanning electron microscopy revealed that the axons perfused with a F^--containing solution had an excitable membrane covered with a highly organized fibrous layer on the axoplasmic surface (Fig. 3). At least two types of filaments were distinguishable by their diameters. Under the transmission electron microscope, these fibers were identified as microtubules, neurofilaments and presumably actin filaments. The thickness of this fibrous layer varied from axon to axon depending on the time and the concentration of the protease in the initial perfusion solution. It was about 1 to 2 μm when the protease treatment was limited to the extent that the action potential remained normal.

Axons Perfused with Chaotropic Anions

The pressure difference across the axon membrane was initially set at 18 cm H_2O. Under this condition, the axon perfused with a solution containing 360 mM KF exhibited stable resting (−51 mV) and action (110 mV) potentials. After a replacement of 360 mM KF with 360 mM KCl, the resting potential was increased or unaltered for about 5 min. The

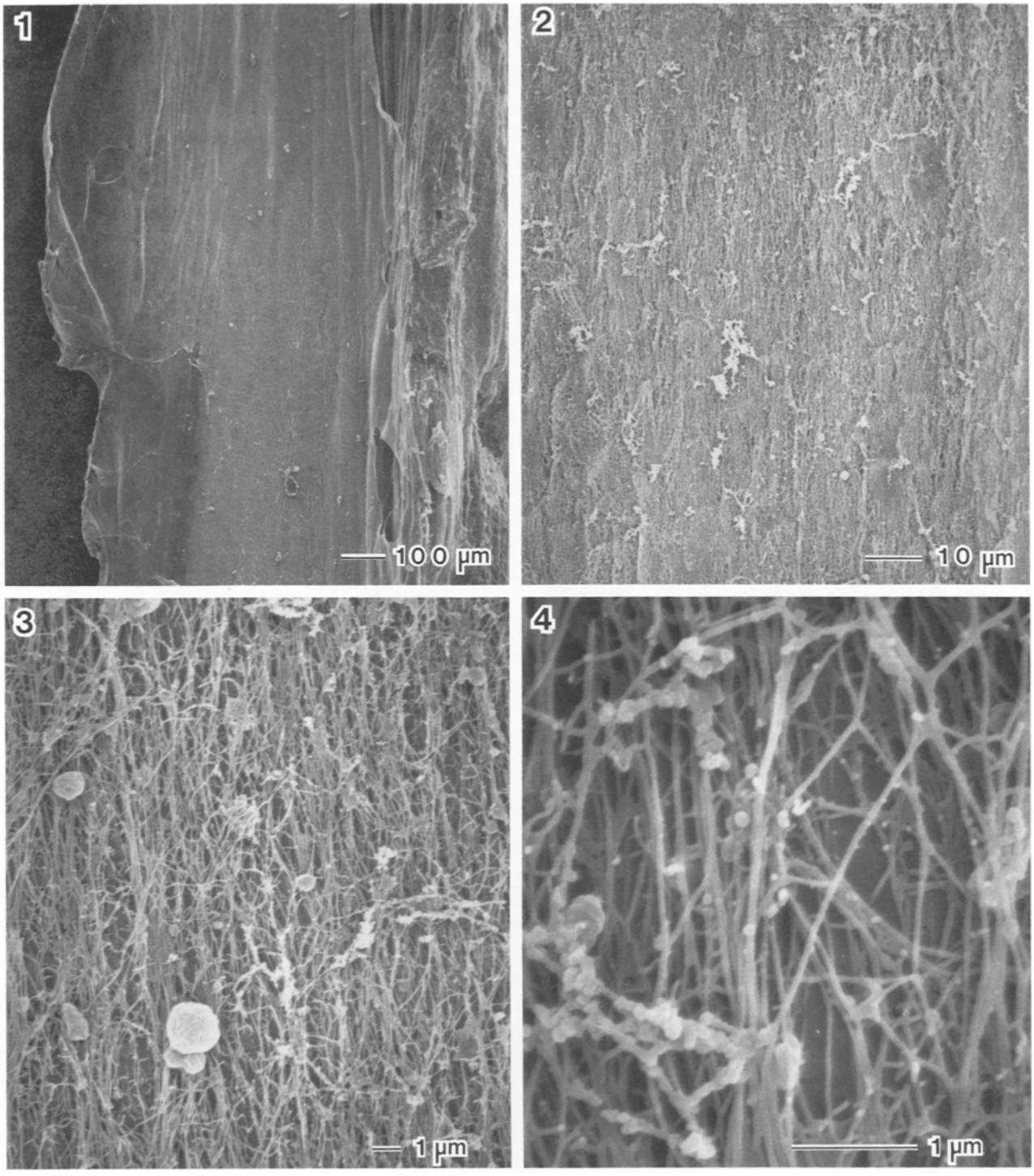


Fig. 3. Axoplasmic surface of an axon perfused continuously with a 360 mM KF solution for 50 min. The amplitude of the action potential was 105 mV when measured immediately before the fixation. The intracellular pressure was kept at 14 cm H_2O and the intracellular solution was allowed to flow

action potential, however, gradually decreased (Fig. 4). The resting membrane potential, then shifted gradually in the depolarizing direction, and the action potential became progressively small. The action potentials were suppressed when the membrane depolarized by 10 to 15 mV from the initial resting level. When the intracellular pressure was further maintained at a high level, the suppres-

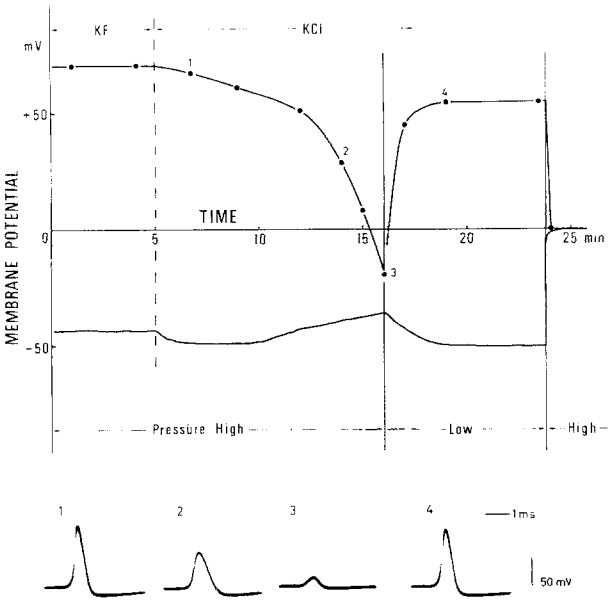


Fig. 4. Reversible effects of the intracellular pressure on the resting and the action potentials of a KCl-perfused axon. The ordinate represents the peak values of the action potential and the resting potential level. The origin is the potential level of the external medium. The abscissa represents the time. The major salt (360 mM KF) in the intracellular medium was replaced with 360 mM KCl at the time indicated by the broken line. The value of the intracellular pressure was about 14 cm H_2O in the area indicated as high, and about 0 cm H_2O in that indicated as low. The action potentials in the lower panel were obtained at times indicated by the corresponding numbers in the diagram

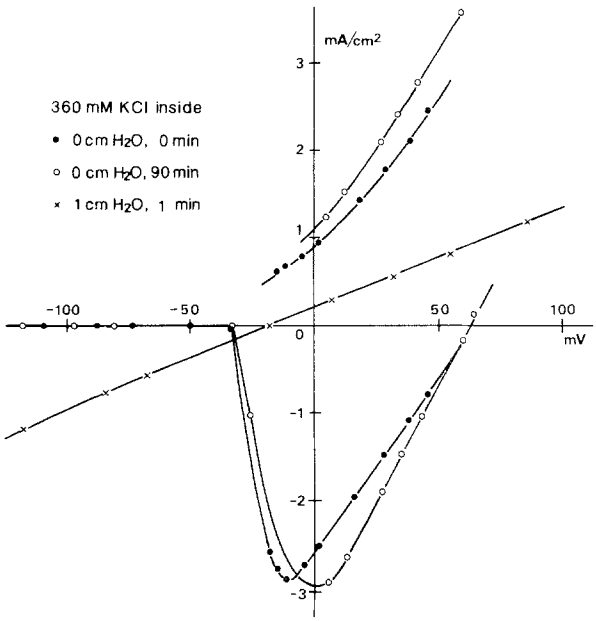
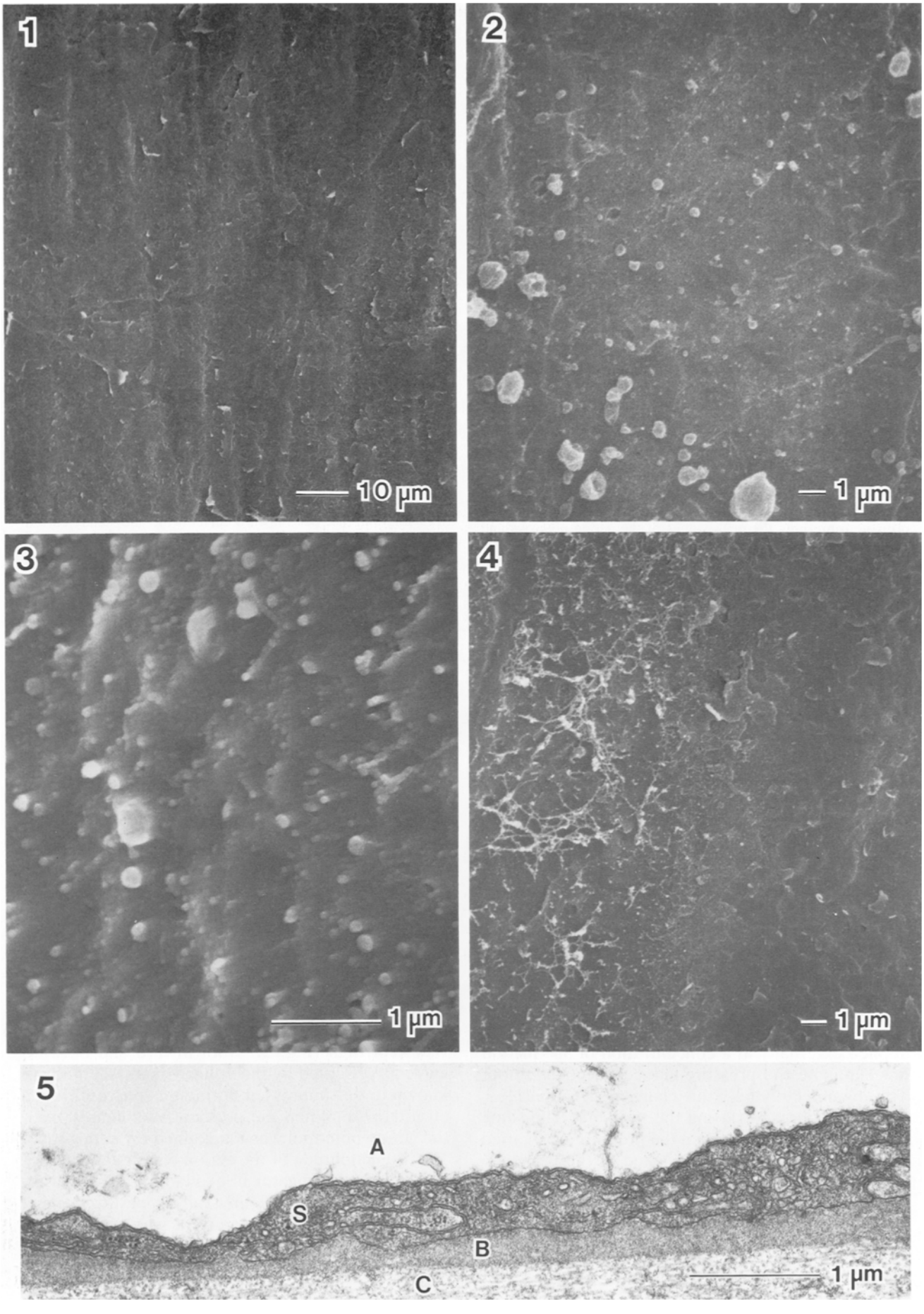


Fig. 5. Pressure sensitivity of the axon perfused with a 360 mM KCl solution. The peak inward current, the late steady current and the leakage current measured by the voltage-clamp method were plotted. The ordinate represents the membrane current; the negative region indicates the inward direction. The abscissa represents the voltage of the clamping pulse. The origin is the potential level of the external medium. (These conditions hold for all other figures of *I–V* curve.) The holding potential was −49 mV. Closed circles represent data obtained immediately after the introduction of 360 mM KCl solution inside. Open circles represent data obtained after a 90-min treatment of the interior of the axon with a 360 mM KCl solution without applying pressure. Crosses indicate data obtained 1 min after raising the intracellular pressure by 1 cm H_2O. At this pressure, the holding potential (= the resting potential) was −18 mV

sion of excitability became irreversible. However, when the intracellular pressure was reduced, before the irreversible suppression was reached, the resting and the action potentials recovered almost completely to their original levels in 5 to 10 min. After this recovery, raising the intracellular pressure again to the initial level resulted in a loss of both the resting and action potentials in a few seconds. The membrane depolarization induced by raising the intracellular pressure was associated with a marked reduction in membrane resistance. A fall of the membrane resistance from 1 kΩ cm^2 to 200 Ωcm^2 was observed when the membrane depolarized by 10 mV from the original resting level. Tetrodotoxin applied externally at a concentration of 300 nM did not eliminate the pressure-induced depolarization. The diameter of the axon changed by about 15% upon lowering and raising the pressure. The same results were obtained when the pressure was varied with the flow of solution blocked at the outlet pipette.

Properties very similar to those described above could be observed in axons perfused with a solution containing 360 mM KBr. In this case, the pressure-sensitive state appeared earlier: usually 5 to 10 min after the onset of internal perfusion. With I^- as the internal major anion, a loss of excitability was observed when the intracellular pressure was raised only a few minutes after the onset of perfusion. In addition, when excitability was lost, it could not easily be regained by reducing the intracellular pressure.

Axons perfused with a KCl solution lost excitability gradually when the intracellular pressure was kept high (Fig. 2). Both inward and outward currents observed by the voltage-clamp method are known to be suppressed during this process (Inoue et al., 1976). When the pressure was almost zero, the action potential was maintained for a long time. Yet, the amplitude of the action potential might not be a sensitive indicator of excitability. Thus, in order to establish that Cl^- *per se* was inert to channel activities, the membrane current was examined by the voltage-clamp method in the axon exposed in-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tracellularly to a KCl solution. The mechanical stress due to the intracellular pressure was avoided as much as possible. When a 360 mM KCl solution was introduced into the axon the intracellular pressure was raised to 15 cm H_2O for 2 to 3 min; then, it was reduced to a level near zero so that depolarization did not proceed. A low negative pressure was applied occasionally for brief periods of time in order to maintain the resting potential. Eventually, axons collapsed in many cases. It was possible to maintain the inward and outward current at the initial level for more than 90 to 100 min (Fig. 5). During this period, the membrane depolarized and the inward current decreased very sensitively when the intracellular pressure was raised slightly. In fact, a raise in intracellular pressure only by 1 cm H_2O immediately resulted in a significant depolarization and a complete suppression of the transient inward current (Fig. 5). The transient inward current could not be observed though the holding potential was varied in a wide range. When the intracellular pressure was adjusted carefully by monitoring the membrane potential, full excitability was maintained for more than 1 hr even in axons perfused with a 360 mM KBr solution. As shown in Fig. 1, these were the times when the axon lost excitability completely if the pressure was kept high. These results demonstrated the extreme sensitivity of the axon perfused with chaotropic anions to mechanical expansion or stress.

The cytoskeletal structure was examined by electron microscopy. Axons treated with a solution containing 360 mM KCl for 1 hr were fully excitable when the intracellular pressure was kept low. Immediate fixation of these axons revealed that the internal surface of the axolemma was free of cytoskeletal fibers (Fig. 6). Vesicles of various sizes were scattered on the axolemma. Scanning electron micrographs showed that the dissolving effect of Cl^- was very uniform on a wide area (more than 90%) of the axolemma. In a few cases, the borderlines between individual Schwann cells could be clearly traced as the axolemma formed ridges or foldings along them. Schwann cell nuclei could be outlined also through the axolemma. When axons were fixed after a shorter period (30 min) of KCl treatment, the cytoskeletal fibers partly remained, but their appearances were much deformed in comparison to those in Fig. 3. These changes were observed in axons treated without applying high intracellular pressure.

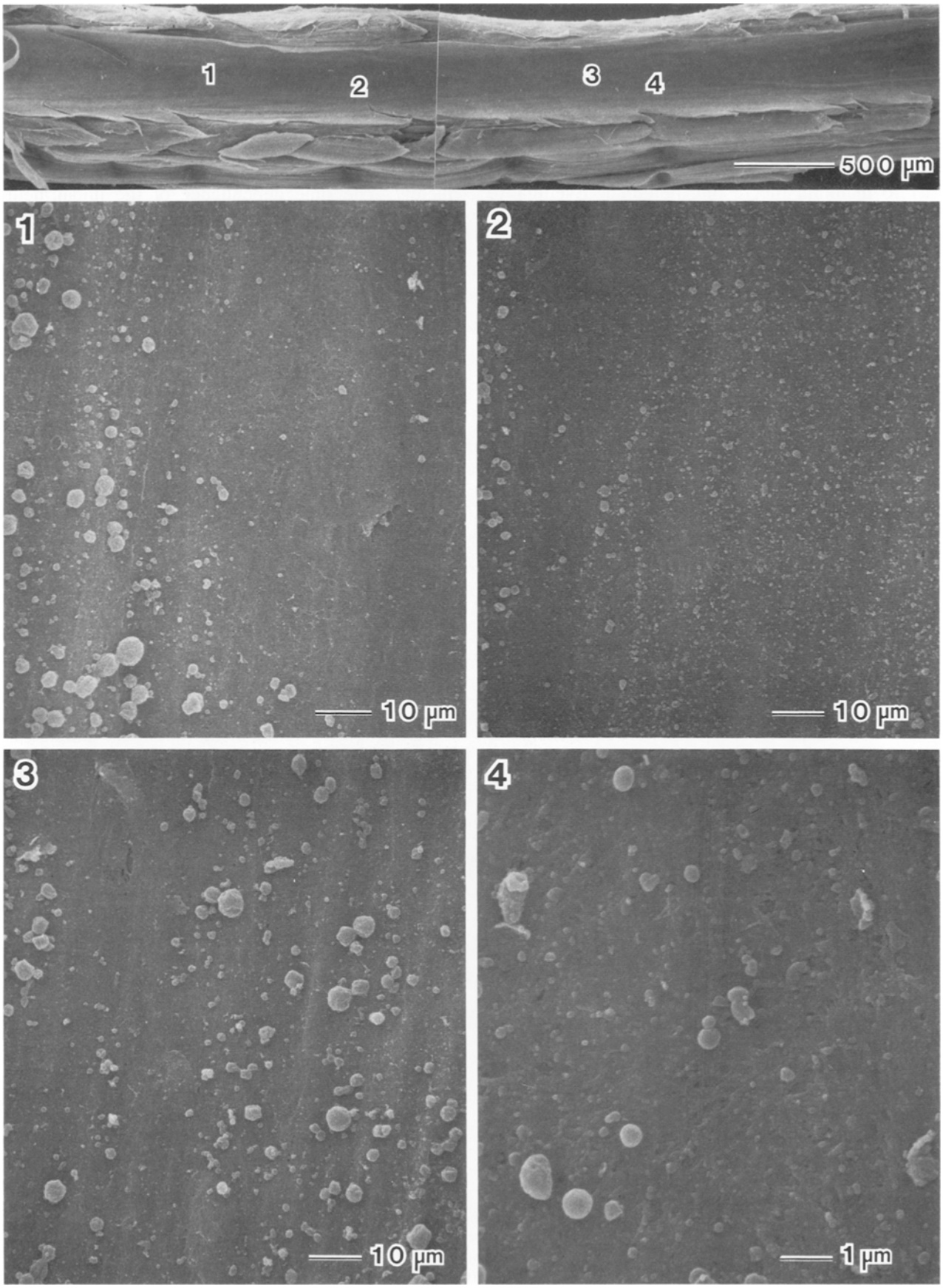
Axons treated with a solution containing Br^- as the major anion showed structural changes on the internal surface of the axolemma, similar to those described above (Fig. 7). However, the time from the onset of perfusion to disappearance of the cytoskeletal network was much shorter. It took only 5 to 10 min for the fibrous structure to be markedly deformed and 15 to 25 min to be dissolved completely. The time dependence of the impairment suggested that the loss of cytoskeletons was not due to an incomplete fixation but due to the treatment with chaotropic anions. Use of I^- as the major anion in the perfusion solution also resulted in a rapid disappearance of the cytoskeletal structure. In addition, the axolemma was partially disrupted. Under the transmission electron microscope, Schwann cells in these parts appeared torn and emptied of their contents. These structural changes of Schwann cells were observed also in the axon perfused with a KCl solution under a high intracellular pressure.

Effects of Colchicine and Pressure

Colchicine is claimed to suppress the activity of Na channel (Matsumoto & Sakai, 1979*a*,*b*; Matsumoto et al., 1980, 1984*a*,*b*; Chang, 1983) in the squid axon. If this effect of colchicine is due to an alteration of cytoskeletal structure, it might depend on the mechanical stress applied on the membrane. Thus, the effect of colchicine on the electrophysiological properties of the membrane was examined in the presence and absence of mechanical stress, i.e., a high intracellular pressure.

When the intracellular medium was 180 mM KF solution, raising and lowering the intracellular pressure between 0 and 15 cm H_2O had little effect on the amplitude and the threshold of the action potential. The addition of colchicine to the internal medium at the concentration of 5 mM while maintaining the intracellular pressure at 15 cm H_2O resulted in a rather rapid decrease in amplitude and increase in threshold of the action potential (Fig. 8). The action potential was almost completely suppressed in 10 min. However, reducing the intracellular pressure to zero reversed these changes completely in spite of the intracellular presence of colchicine. Under a low pressure, axons which were yellowish in color, confirming the continued presence of colchicine, generated normal action potentials.

Fig. 6 (*facing page*). (1)–(3) Scanning electron micrographs showing the internal surface of the axolemma treated intracellularly with a 360 mM KCl solution for 1 hr. The intracellular pressure was kept at 0 cm H_2O. (4) The axon was treated with the 360 mM KCl solution for 30 min. (5) Transmission electron micrograph showing the cross-section of an axon treated similarly to (4). *A*, Axoplasmic space. *S*, Schwann cell layer. *B*, Basal membrane. *C*, Collagen layer. In all cases, the axon exhibited action potentials 100 to 110 mV in amplitude until the moment of fixation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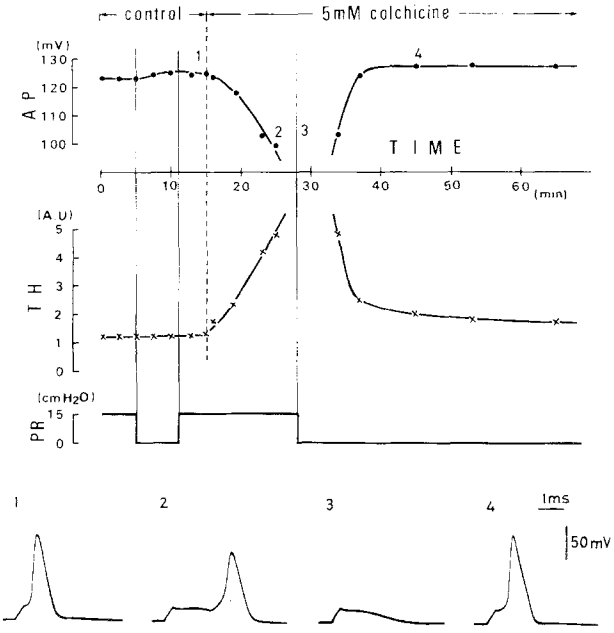

Fig. 8. Effect of colchicine and the effect of the intracellular pressure on the action potential (*AP*) and the threshold (*TH*). The threshold level is expressed in arbitrary units. The intracellular pressure (*PR*) was varied between 0 and 15 cm H_2O. The intracellular solution contained 180 mM KF and some K phosphates during the period indicated as control. Then, during the period after the broken line, the solution additionally contained 5 mM colchicine. The action potentials shown in the lower panel were obtained at the time indicated by the corresponding number in the diagram. They were induced by stimulating the axon with 3-msec depolarizing pulses applied through an internal wire electrode.

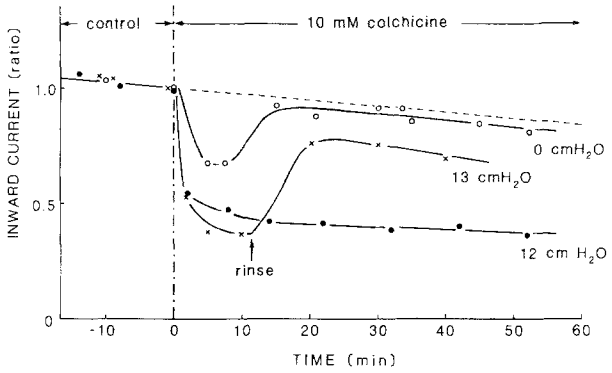

Fig. 9. Effect of colchicine on the peak inward current examined under various intracellular pressures. During the period indicated as control, the intracellular solution contained 180 mM KF and some K phosphates. At time zero, 10 mM colchicine-containing solution was introduced intracellularly. The intracellular pressure was maintained at the level indicated throughout each measurement except for the case of 0 cm H_2O where the pressure was temporarily raised to 7 cm H_2O for 2 min upon introduction of colchicine. Colchicine was removed at the time indicated by the arrow in the case of 13 cm H_2O

The membrane currents were examined by applying a step depolarization under the voltage-clamp condition (Fig. 9). In a control solution, the peak inward current decreased very gradually. Introducing 10 mM colchicine into the intracellular space led to two different results depending on the intracellular pressure. For example, at a pressure of 12 cm H_2O, 10 mM colchicine decreased the peak inward current within the initial 10 min to half the control (untreated) levels which was constant as long as the same pressure was applied. Removal of colchicine under these conditions returned the inward current to the control levels. However, after the intracellular introduction of 10 mM colchicine and reduction of the pressure to 0 cm H_2O, the inward current decreased only temporarily and later returned to the control levels even in the presence of intracellular colchicine.

With *I–V* curves, the pressure sensitivity of the Na conductance was much higher in the presence of colchicine than in its absence (Fig. 10). The reduction of the conductance appeared without a significant change in the reversal potential. This indicated that the expansion of the membrane affects the Na channel in the activation mechanism only. The K conductance was also sensitive to the intracellular pressure. The excitability revealed by the *I–V* curve was not affected by intracellular colchicine unless the intracellular pressure was kept high (Fig. 11). Colchicine alone did not affect the ionic channel directly.

Effects of lumicolchicine were examined by using a 0.1 mM suspension as the internal medium since its water solubility was rather low. The effect was similar to that observed with high concentrations (1 to 10 mM) of colchicine. The effects of extracellular colchicine were examined in five intact (unperfused) axons mounted in the chamber with a minimal amount of mechanical stress. The internal electrode was only a 50-μm Pt wire. The amplitude and the maximum rate of rise of action potentials were recorded every 7 sec automatically under the control of a microcomputer. The continuous plotting of these data provided a very sensitive measure for the effects elicited by chemical reagents. After extracellular application of 10 mM colchicine, no

Fig. 7 (*facing page*). Scanning electron micrographs showing the internal surface of the axolemma treated intracellularly with a 360 mM KBr solution for 17 min. The intracellular pressure was set at 0 cm H_2O. Each numbered photograph shows a high magnification view of the field indicated by the corresponding number in the figure shown on the top. The axon exhibited the action potential 106 mV in amplitude until the moment of fixation

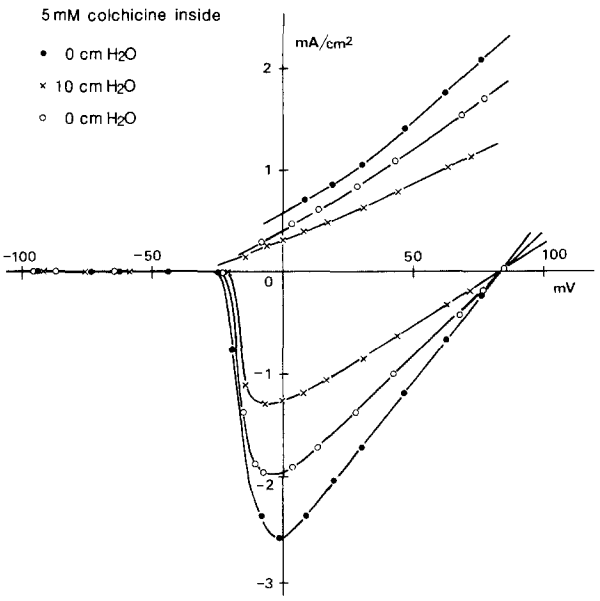

Fig. 10. Pressure sensitivity of the *I–V* curve in the axon treated with 5 mM colchicine. Colchicine was introduced by intracellular perfusion 20 min before measurements. Each *I–V* curve was obtained 12 min after changing the intracellular pressure

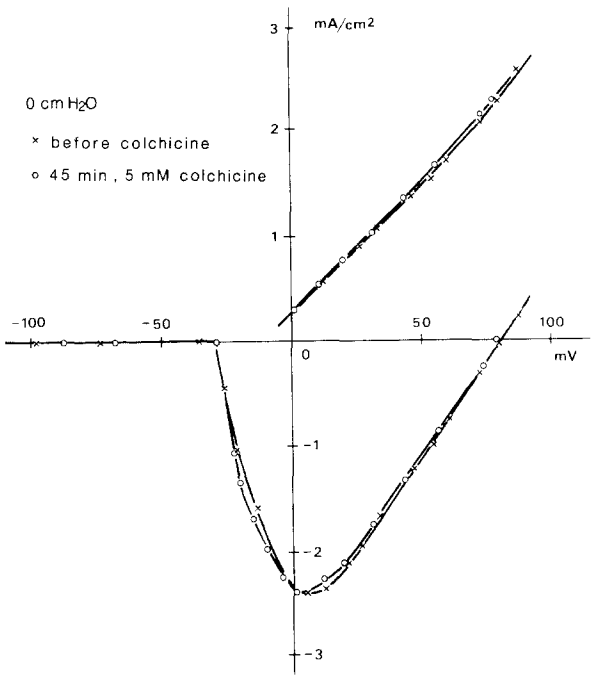

Fig. 11. Elimination of the colchicine effect on the *I–V* curve by reduction of the intracellular pressure. The intracellular pressure was kept at 0 cm H_2O except for a 2-min period necessary for introducing 5 mM colchicine. (×) Control obtained before application of colchicine. (○) Data obtained 45 min after application of colchicine

sign of reduced excitability was observed for more than 1 hr.

The effects of colchicine on the cytoskeletal structure were not as we had expected. The axon was fixed 30 min after intracellular perfusion with a 10 mM colchicine-containing solution. Microtubules and other filaments located near the internal surface of the axolemma were not affected by this treatment (Fig. 12). The results were the same irrespective of whether the intracellular pressure was high or low when colchicine was applied. Transmission electron microscopy disclosed many microtubules. Their density (about 30 microtubules per μm^2 of the cross area) was not significantly different from that of control. Thus, there is a clear discrepancy between this finding and the previous observations (Matsumoto et al., 1983).

Cytoskeletons Solubilized in the Perfusate

The resistance of microtubules to colchicine was further examined by biochemical analyses of cytoskeletal proteins solubilized during intracellular perfusion. The perfusate was collected at the outlet pipette every 15 min, and its protein composition was examined quantitatively by SDS polyacrylamide gel electrophoresis (Fig. 13). Initially, a large quantity of proteins was released from the axon interior into the perfusate. As the perfusion continued with a KF solution, the quantity decreased gradually and reached a low level as shown previously (Yoshioka et al., 1978; Pant et al., 1978). Addition of 10-mM colchicine to the KF solution did not enhance the release of proteins such as tubulin, actin and neurofilament. No increase in protein release was detected even after perfusion for 30 min; a period long enough for colchicine to complete its effect on the physiological function of the axolemma. However, the intracellular application of a KCl solution resulted in a rapid and large release of these cytoskeletal proteins. These observations were repeated in 5 axons.

Discussion

Chaotropic anions such as Cl^-, Br^- and I^- enhance depolymerization of F actin (Guba, 1950), microtubules (Sakai & Matsumoto, 1978) and cytoskeletal proteins in general (Inoue et al., 1976; Baumgold et al., 1981). Intracellular perfusion with solutions containing these chaotropic anions always resulted in a rapid loss of excitability (Inoue et al., 1976; Metuzals & Tasaki, 1978; Yoshioka et al., 1979; Matsumoto et al., 1984*a*). These findings suggested that cytoskeletal proteins were involved in the mo-

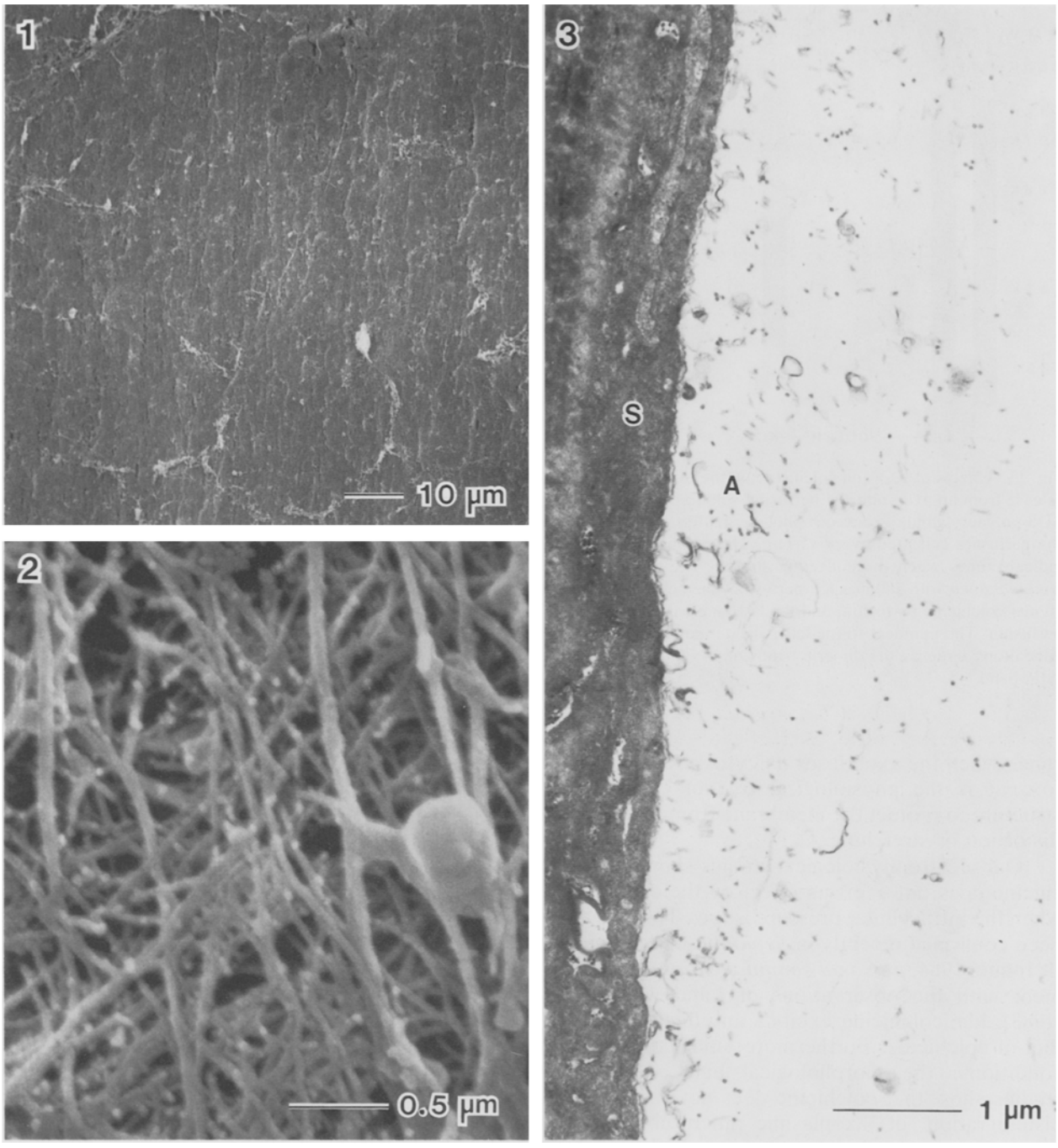

Fig. 12. Electron micrographs of the cytoskeleton in axons perfused with a solution containing 180 mM KF, 10 mM colchicine and some K phosphates for 30 min. (1) Scanning electron micrograph obtained with a low power magnification. (2) Scanning electron micrograph obtained with a high power magnification. (3) Transmission electron micrograph of the cross-section of an axon. *S*: Schwann cell layer. *A*: Axoplasmic space

lecular machinery of the ionic channels. Particularly, microtubules were speculated to be related to the gating mechanism of Na channels (Matsumoto et al., 1984*b*). The present study, however, has demonstrated that the fibrous proteinaceous network underneath the membrane can be removed completely without influencing the excitability. This is attained by adjusting the intracellular pressure. The time when the axon becomes extremely sensitive to intracellular pressure is well beyond the

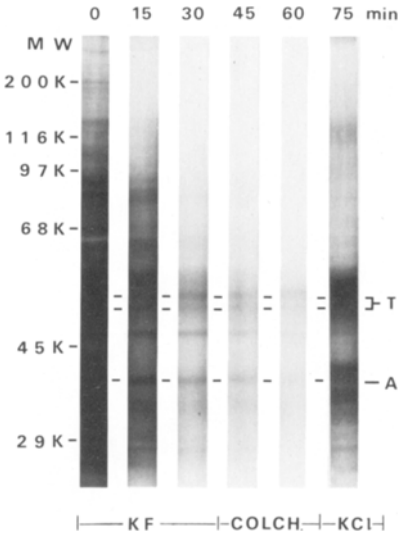

Fig. 13. Effects of KCl and colchicine on the rate of the protein release from an axon into the perfusion solution. Each lane is a SDS-polyacrylamide gel electrophoresis pattern of proteins in the perfusate collected every 15 min. The numbers on the top indicate times when the collection started. The initial 3 lanes were obtained with 180 mM KF perfusion, the next 2 lanes with 10 mM colchicine perfusion, and the last lane with 360 mM KCl perfusion. The numbers to the left indicate molecular weight in kilodalton. *T* represents dimeric bands of tubulin. *A* represents actin band

range when the axon loses its cytoskeleton. Therefore, it is the physiological role of cytoskeletal structure to protect the membrane from mechanical distortion or stretching.

Our electrophysiological studies show that colchicine does not exert an effect on the excitability when the intracellular pressure is low. This has also been confirmed recently in *Sepiotheusis lessoniana* (I. Inoue, 1984, *personal communication*). In agreement with the observations of Landowne et al. (1983), lumicolchicine exhibits an effect similar to that of colchicine. Furthermore, under the present conditions, the morphological and biochemical studies show that colchicine does not enhance the disintegration of axoplasmic microtubules. All these observations indicate that axoplasmic microtubules *per se* are not functionally involved in the molecular machinery of ionic channels. Colchicine may either reduce the ability of the cytoskeleton to protect the membrane against the mechanical stress, or directly alter the structural stability of the membrane by binding nonspecifically to membrane sites (Stadler & Franke, 1974). In either case, it is clear that the mechanical stress due to the high intracellular pressure influences the activity of ionic channels.

The adverse effects of intracellular pressure on the excitability was first pointed out by Baker et al. (1962). As shown in our results, the intracellular pressure does play a key role in relating cytoskeletons to the excitability. Some previous results will require re-interpretation with consideration of mechanical stresses loaded on the experimental preparations. Matsumoto and Sakai (1979*b*) showed that the excitability of the axon membrane, once suppressed by intracellular perfusion with solutions unfavorable for microtubule assembly, can be restored by perfusing the axon with a solution containing tubulin and its polymerizing reagents. We wish to suggest two possible interpretations for this finding. One is that the reassembly of microtubules in the intracellular space reduced the susceptibility of the axon to the intracellular pressure by repairing the membrane-associated cytoskeletons. The other is that reassembly reduced the intracellular pressure by increasing the viscosity of the solution and by blocking the inlet pipette. Both mechanisms may facilitate the recovery of axon excitability. However, neither support the conclusion that microtubules play an essential role in the ion-channel mechanism responsible for production of the action potential.

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