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Microtubules inside the Plasma Membrane of Squid Giant Axons and their Possible Physiological Function

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Summary. The effects of application of the microtubule-disassembling reagents to squid giant axons upon resting potential, the height of the propagated action potential, and the threshold to evoke action potential were studied using colchicine, podophyllotoxin, vinblastine, griseofulvin, sulfhydryl reagents including NEM, diamide, DTNB and PCMB, and Ca^{2+} ions. At the same time, the effects of concentrations of K halides and K glutamate on the above physiological properties were studied in comparison with *in vitro* characteristics of microtubule assembly from purified axoplasmic tubulin.

It was found that there was good correlation between conditions supporting maintenance of membrane excitability and microtubule assembly. The experiments suggest that associated with the internal surface of the plasma membrane there are microtubules which regulate in part both the resting and action potentials.

Recent studies have revealed that the axoplasm of squid giant axon contains a large amount of microtubules and neurofilaments (Huneeus & Davison, 1970; Davison & Huneeus, 1970; Gainer & Gainer, 1976; Sakai & Matsumoto, 1978). Microtubules in the neuron have been believed to function in axonal transport (James *et al.*, 1970; Abe, Haga & Kurokawa, 1973).

In electrophysiological studies, the perfusion experiments have shown that the electrical excitability of squid giant axons could be suppressed as a result of releasing a substantial amount of axonal protein (Inoue *et al.*, 1976; Yoshioka *et al.*, 1978). Electron microscopically, Metuzals and Tasaki (1978) showed that a three-dimensional network of interwoven filaments consisting partly of an actin-like protein is firmly attached to the axolemma and might have a role in some aspect of excitability. Upon analyzing axonal proteins in perfusate with SDS-polyacrylamide gel electrophoresis, Gainer *et al.* (1974), Inoue *et al.* (1976), and Takenaka

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et al. (1976) concluded that a 12,000-dalton protein was released after repetitive electrical stimulation of the axon or by potassium depolarization. However, quite recently, Pant *et al.* (1978) concluded that an appreciable amount of a 45,000-dalton protein, in addition to the 12,000 daltons, presented in the perfusate from the stimulated axon or the axon exhibiting long-lasting action potential, while a 68,000-dalton protein dominated in the perfusate from the depolarized axon. Furthermore, Yoshioka *et al.* (1978) found that suppression of axonal excitability was associated with the release of a 56,000-dalton protein. It was recently found that the order glutamate $\sim$ F > Cl > Br > I to support axoplasmic microtubule assembly (Sakai & Matsumoto, 1978) was qualitatively in agreement with that for the maintenance of the membrane excitability determined by Tasaki, Singer and Takenaka (1965).

This paper deals in more detail with the effects of microtubule depolymerizing reagents upon physiological parameters including resting potential, action potential, and threshold to maintain the excitability of squid giant axons. The effects of concentration of K halides and K glutamate upon the physiological properties are also given in more detail in comparison with the *in vitro* characteristics of axoplasmic microtubule assembly.

Materials and Methods

Intracellular Perfusion of Squid Giant Axons

Giant axons of the squid (*Doryteuthis bleekeri*) were used. Squids were collected in Sagami Bay, transported to, and kept alive in a special aquarium in the Electrotechnical Laboratory (Matsumoto, 1976). The internal perfusion of the axon was performed principally by the technique developed by Tasaki (1968). The only difference is that in our method the inlet glass cannulae was inserted into the outlet glass cannulae at one extreme end of the perfusion zone to remove the axoplasm uniformly from the entire perfusion zone, while in the original method this was done right in the middle of the zone. The length of the perfusion zone was usually 15 mm. A glass pipette Ag-AgCl electrode, 80 μm in diameter and filled with 3 M KCl-agar gel, was used to record the electric potential in the middle of the perfusion zone inside the axon. A bright silver wire, 25 μm in diameter, was inserted inside the glass pipette of the recording electrode up to 0.2 mm before the tip to reduce the capacitance (Hodgkin & Katz, 1949; Chandler, Hodgkin & Meves, 1965). An Ag-AgCl wire electrode, 50 μm in diameter, bare for 1 cm length and the other portion coated with enamel, was used as a stimulating electrode. Stimulation shocks were delivered internally only at threshold measurements. The threshold was defined as the amplitude of stimulating current pulse enough to barely evoke action potential, where the current pulse width was fixed between 1.25 and 1.45 msec. Otherwise, they were given externally. A calomel electrode immersed in the external medium was used as the reference point for potential measurements. The external medium was grounded through a large coil of Ag-AgCl wire.

Internal perfusion solutions consisted of three components: KF, K glutamate or KCl

was used as salt, K phosphate as a buffer and 12% (vol/vol) glycerol solution as an osmotic carrier. In the case of internal application of Ca ions (*see Results*), 33 mM K-HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) was used as a buffer (pH 7.2). The standard perfusion solution consisted of 360 mM KF, 40 meq/liter K ion containing phosphate buffer and glycerol. These solutions were prepared by the method developed by Tasaki (1968). All the perfusion experiments started after the axon was initially perfused with the standard solution containing 0.05 mg/ml protease VII for 30–60 sec.

Others

Purification of microtubule proteins and assay of microtubule assembly were described elsewhere (Sakai & Matsumoto, 1978). All the physiological experiments described were carried out at room temperature (15–20 °C). Vinblastine, podophyllotoxin, colchicine, griseofulvin, diamide, N-ethylmaleimide (NEM), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), *p*-chloromercuribenzoic acid (PCMB), and dithiothreitol (DTT) were purchased from Sigma Chemical Co., St. Louis, Mo. Lumicolchicine was prepared by irradiating colchicine with a 500 W Xe lamp. The lamp illuminated a petri-dish containing 2.5 mM colchicine dissolved in the standard perfusion solution for half an hour and was placed approximately 1 m away from the dish. All these reagents except griseofulvin were used after being directly dissolved in the standard perfusion solution containing 400 meq/liter K ion. Griseofulvin was first dissolved in N,N-dimethyl formamide (DMF) to make a 200 mM solution. Then the DMF solution was mixed with the standard perfusion solution to prepare appropriate concentration of griseofulvin.

Results

1. Inhibitors for Microtubule Assembly and Lumicolchicine

Colchicine. In the internal application of 2.5 mM colchicine, gradual changes were observed in electrophysiological properties for a period of 50 min. The amplitude of action potential was slightly reduced once but recovered to the original 20 min after the onset of perfusion (Fig. 1 *A*). On the other hand, the threshold rapidly increased at first, and then gradually increased with time. The rapid increase in the threshold corresponds to the decrease in the amplitude of action potential. The reciprocal relation between the decrease in the amplitude of action potential and the increase in the threshold was evident for the axon perfused internally with 10 mM colchicine solution (Fig. 1 *B*). The resting potential was unchanged by the internal application of 2.5 mM colchicine but reduced by about 5 mV 50 min after application of 10 mM colchicine.

The effect of external application of 2.5 mM colchicine on the electrophysiological properties was qualitatively similar to that of internal application except that the rapid increase in the threshold and rapid fall of

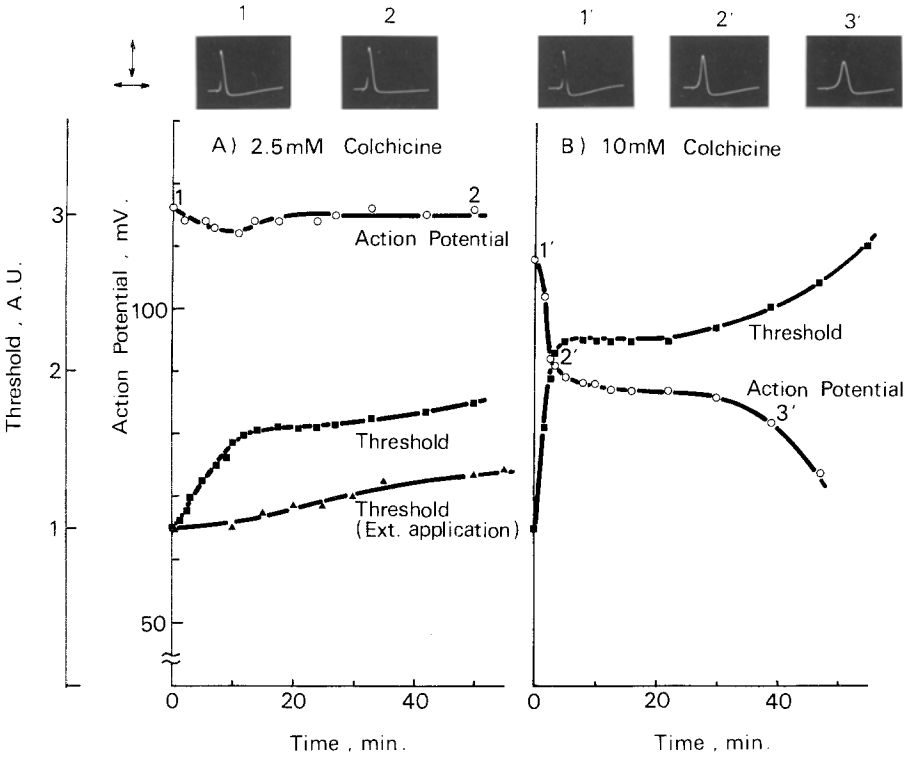

Fig. 1. Effect of colchicine upon the electrophysiological properties. Time course of changes in action potential and threshold during internal or external perfusion with 400 mM KF solutions containing 2.5 mM and 10 mM colchicine were shown in *A* and *B*, respectively. Two and three representative oscillograph records are shown on the top of *A* and *B*, respectively. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

the amplitude of action potential could not be observed, but the threshold gradually increased with time during 10 min after the onset of bathing the axon in natural seawater containing 2.5 mM colchicine (Fig. 1 *A*).

Podophyllotoxin and vinblastine. Internal application of 2.5 mM podophyllotoxin and 0.3 mM vinblastine caused very similar changes in electrophysiological properties of the membrane. The effect of podophyllotoxin was stronger than that of colchicine at the same concentration (Fig. 2). After the excitability was decreased by podophyllotoxin, it was partially recovered just after switching the podophyllotoxin solution to 400 mM KF, but the excitability gradually fell again thereafter. In the case of internal application of vinblastine, an abrupt increase in the threshold was observed at the onset of switching the perfusion solution

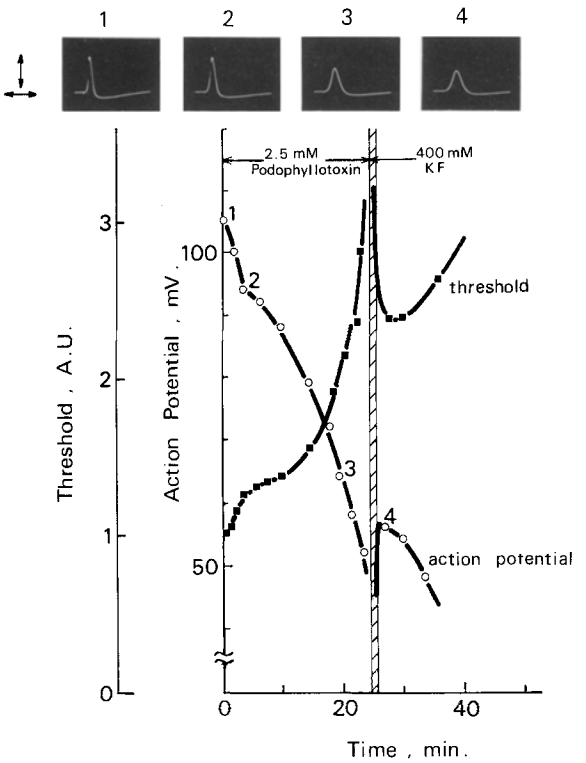

Fig. 2. Effect of podophyllotoxin upon the electrophysiological properties. The figure shows time course of changes in action potential and threshold during intracellular perfusion, first with 400 mM KF solution containing 2.5 mM podophyllotoxin and then with 400 mM KF. The vertical break represents the time necessary to switch the perfusion solutions. Four representative oscillograph records are shown on the top. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

from 400 mM KF to the standard internal solution containing 0.3 mM vinblastine. The reciprocal relation between the threshold and action potential was also obtained. The resting potential was reduced by about 5 mV.

SH reagents. The actions of NEM, diamide, DTNB, and PCMB on the electrophysiological parameters were classified into two groups; one was the effect of internal or external application of NEM and diamide, and the other was those of DTNB and PCMB. In the internal application of 2.5 mM NEM or diamide, the threshold decreased initially and then gradually increased while the amplitude of action potential was monotonically reduced with time (Fig. 3*A*). The external application of 5 mM NEM also caused a similar effect in that the threshold had

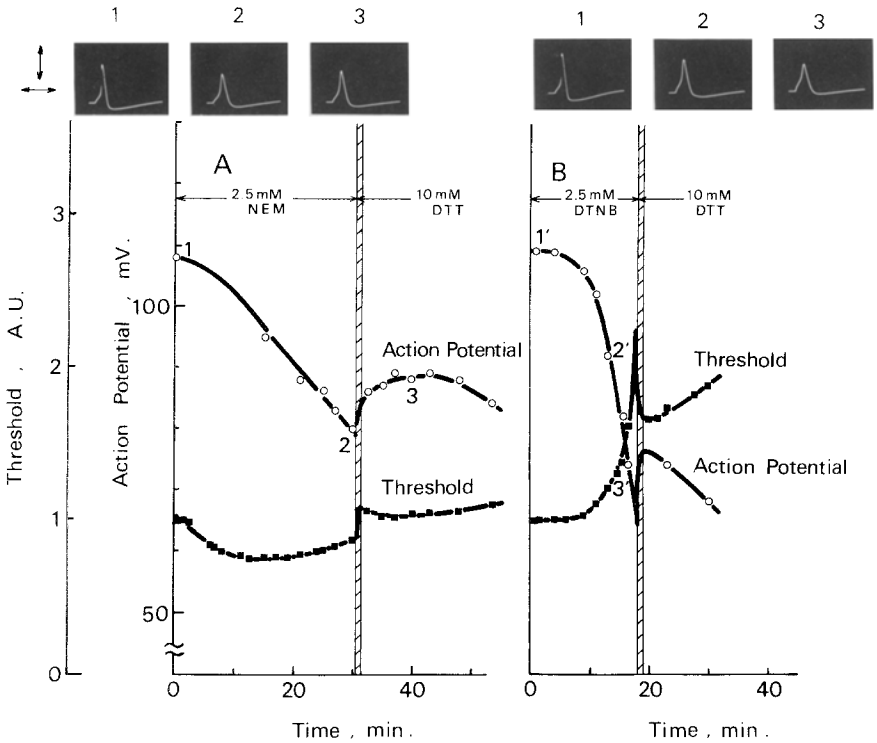

Fig. 3. Effect of NEM and DTNB, upon the electrophysiological properties. The figure shows time course of changes in action potential and threshold during intracellular perfusion, first with 400 mM KF solutions containing 2.5 mM NEM (*A*) and 2.5 mM DTNB (*B*) and then with 400 mM KF containing 10 mM DTT. Three representative oscillograph records are shown on the top in both cases of *A* and *B*, respectively. The number refers to their respective positions in time along the action potential curve. The vertical break represents the time necessary to switch the perfusion solutions. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

a minimum as a function of perfusion time and that the amplitude of action potential was monotonically reduced.

In contrast, the action of DTNB or PCMB was very similar to those observed for colchicine, podophyllotoxin, and vinblastine in that the time course of the variation of the action potential was reciprocally related to that of the threshold. An example of internal application of 2.5 mM DTNB is shown in Fig. 3*B*. The external application of 5 mM DTNB caused changes in electrophysiological properties very similar to those observed with the internal application of 2.5 mM DTNB.

Griseofulvin and lumicolchicine. The internal application of 2.5 mM griseofulvin caused weak effects on physiological parameters (Fig. 4*A*). The resting potential did not change for 50 min. A slight decrease in

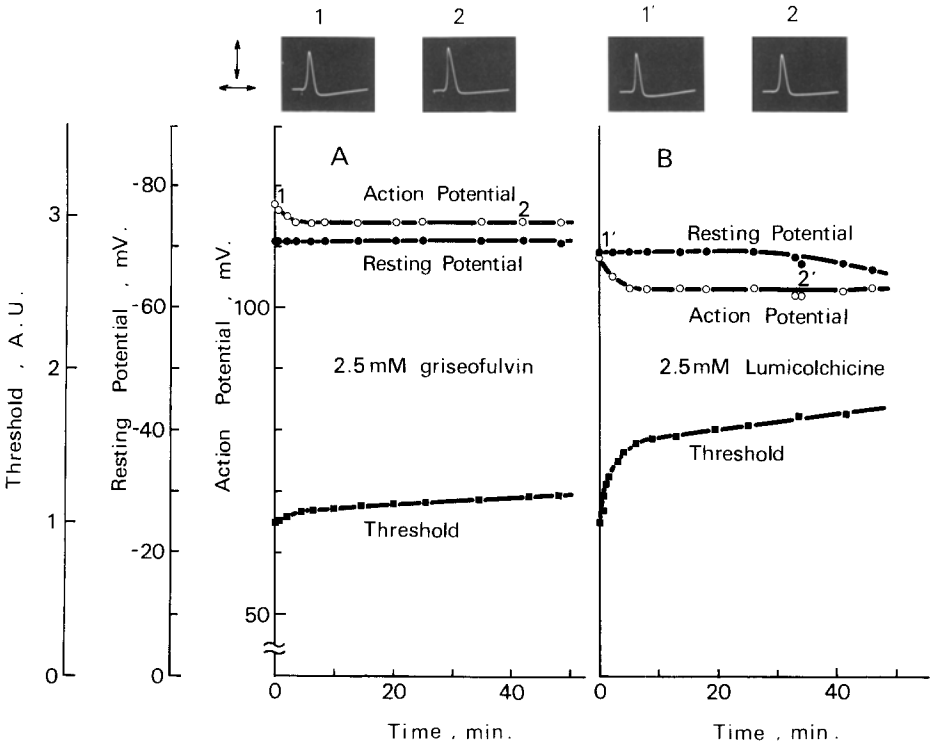

Fig. 4. Effects of griseofulvin (*A*) and lumicolchicine (*B*) upon the electrophysiological properties. The figures show time course of changes in action potential, resting potential, and threshold during intracellular perfusion with 400 mM KF solutions containing 2.5 mM griseofulvin (*A*) and 2.5 mM lumicolchicine (*B*), respectively. Two representative oscillograph records are shown on the respective tops of *A* and *B*. The number refers to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 1 msec and 100 mV, respectively

the amplitude of action potential occurred first rather rapidly, and then it was kept constant. The threshold first increased, corresponding to the decrease in the amplitude of action potential, followed by a gradual increase. For assembled porcine brain microtubules, griseofulvin (1 mM) caused disassembly to occur only by 38% in terms of viscosity decrease at a griseofulvin:microtubule-associated protein molar ratio of 600:1.

The internal application of 2.5 mM lumicolchicine caused electrophysiological changes very similar to those observed by the internal application of 2.5 mM griseofulvin (Fig. 4*B*) in that the amplitude of action potential was kept constant after a rapid decrease at the initial stage of perfusion. The rapid decrease in the action potential correlated in time to a rapid increase in the threshold. Then, the threshold slowly increased. The resting potential was reduced by about 5 mV.

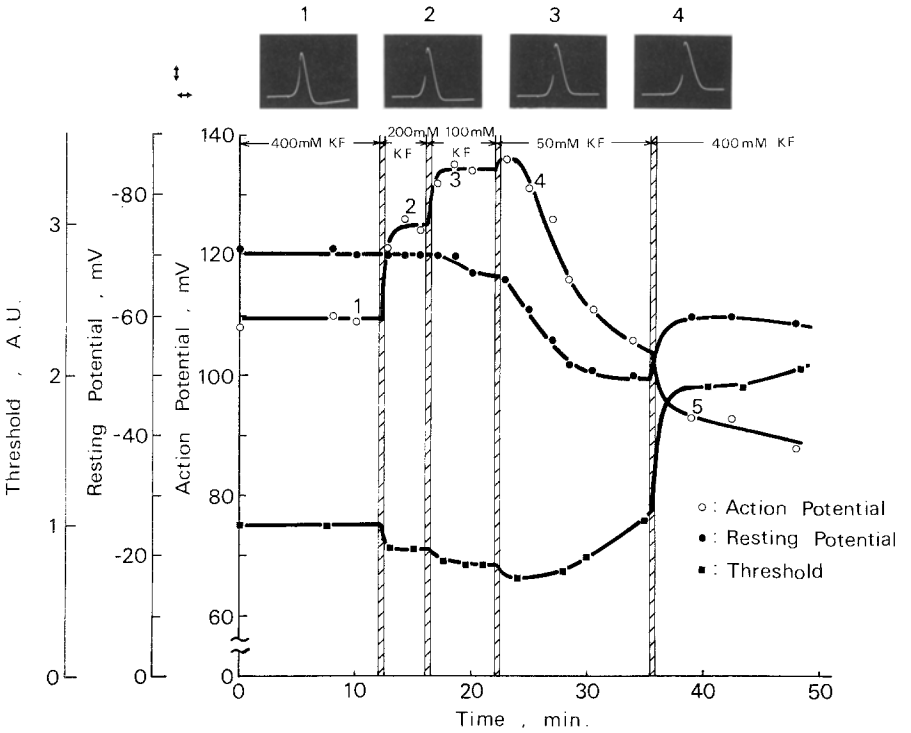

Fig. 5. Time courses of changes in the action potential, resting potential, and threshold during perfusion with a series of KF solutions. The diameter of the axon was 550 μm. The breaks in the figure represent the time necessary to switch the perfusion solutions. Four representative oscillograph records are shown on the top with the numbers which refer to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 0.2 msec and 40 mV, respectively

Ca^{2+} ions. It was well known that internal application of Ca ions disrupted the membrane excitability very quickly (Tasaki, Watanabe & Lerman, 1967; Begenisich & Lynch, 1974; Terakawa, Nagano & Watanabe, 1977). This was also confirmed in this experiment using a perfusion medium containing 400 mM K glutamate and 0.1 mM $CaCl_2$. On the other hand, the internal application of the standard internal solution containing 0.1 mM $MnCl_2$ exhibited no harmful effects upon the excitability during the perfusion for 1 hr.

2. *Anion Effects*

KF and KCl. In the case of the KF or KCl application, the time course of change in the size of the action potential correlated well with

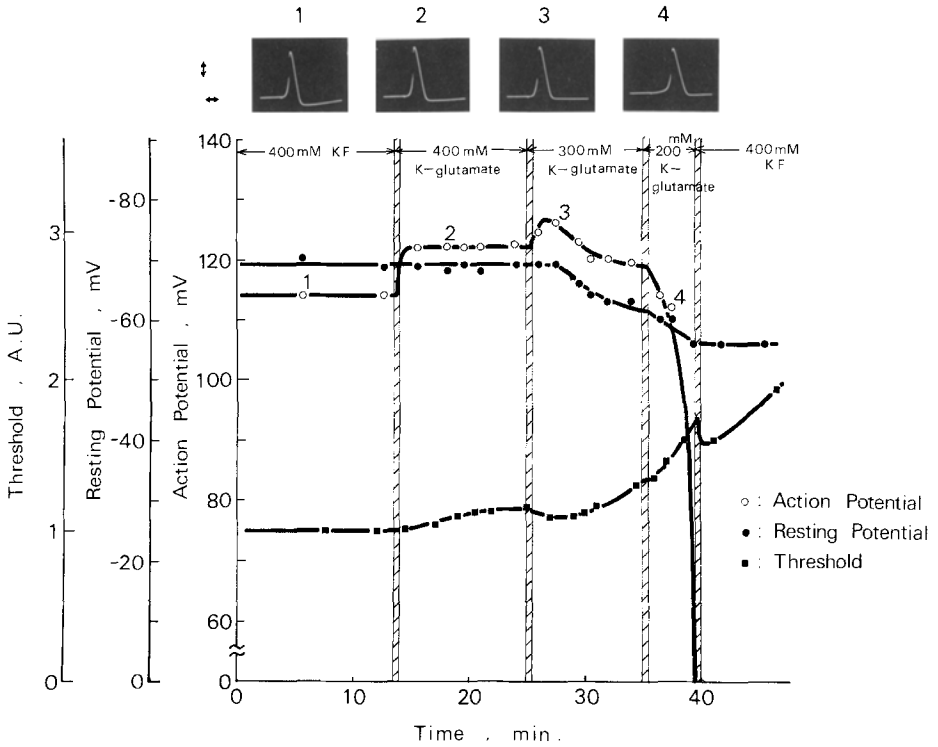

Fig. 6. Time courses of changes in the action potential, resting potential, and threshold during perfusion with 400 mM KF solution and a series of K glutamate solutions. The initial 400 mM KF solution was used to remove the after-effect of the protease pretreatment. The diameter of the axon was 575 μm. The breaks in the figure represent the time necessary to switch the perfusion solutions. Four representative oscillograph records are shown on the top. The numbers refer to their respective positions in time along the action potential curve. The horizontal and vertical bars stand for 0.2 msec and 40 mV, respectively

that of change in the threshold (Fig. 5); i.e., the bigger the size of action potential, the lower the threshold. In the KF application, the axon perfused with more diluted solutions (Fig. 5, from 400 to 100 mM) gave birth to bigger size of action potentials or lower threshold. Figure 5 also shows that the size of the action potential once became bigger consistently just when 100 mM KF was replaced with 50 mM KF, then it became rapidly smaller with time. Furthermore, 25 mM KF caused more rapid and monotonic increase in the threshold with time than that perfused with 50 mM KF solution.

It was generally found that the excitability could not be maintained long for the axon perfused with diluted solution. Therefore, there was a maximum in the size of action potentials or a minimum in the threshold with time during the perfusion. Dependence of the minimum threshold

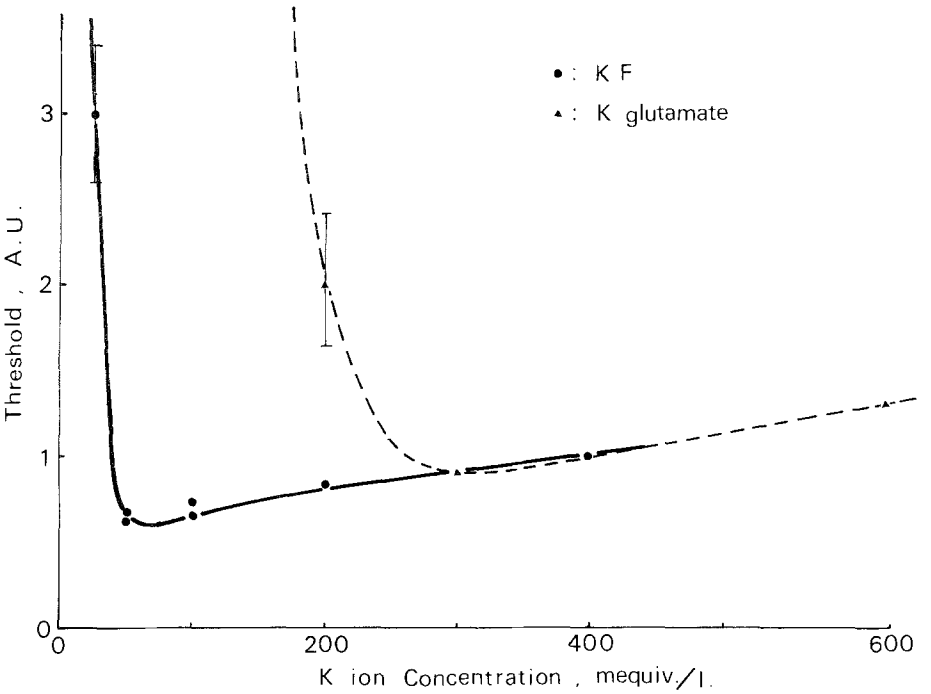

Fig. 7. Dependence of the threshold on K ion concentrations for axons perfused with KF and K glutamate solutions. Closed circles and triangles represent the values in the KF and K glutamate perfusion, respectively. The threshold was obtained as minimum values in the time during perfusion with a solution, and normalized by that obtained during perfusion of 400 mM KF and K glutamate solutions for solid and dotted lines, respectively

thus obtained on the K ion concentration is shown in Fig. 7 as a solid line. The curve has the minimum around a K ion concentration of 75 mM.

From the point of survival time of propagated action potential, diluted K salt solution does not favor the maintenance of excitability. This characteristic was exaggerated in the perfusion of KCl solution, which was less favorable than KF solutions (Tasaki *et al.*, 1965). The replacement of 400 mM KCl with 200 mM KCl caused the size of action potential to increase initially, but it fell rapidly until the conduction of action potential was blocked. The effect on the resting potential of varying the concentration of KF or KCl inside the axon was similar to that observed by Baker, Hodgkin and Meves (1964).

K-glutamate. The overall features observed for electrophysiological parameters upon varying the K glutamate concentration inside the axon

were essentially similar to those found in the KF or KCl application, except for the K ion concentration giving rise to a minimum in regard to the threshold (Fig. 6). This relation is shown in Fig. 7 as a dotted line, where the minimum exists around the 300 meq/liter K ion concentration. The experiments were carried out in the range of K ion concentration below 600 meq/liter. Thus, 300 mM K glutamate was the most favorable to give the biggest size of action potential or the lowest threshold.

Discussion

It has been well established that microtubule assembly and disassembly are governed by many factors such as ionic species, ionic strength, pH, Ca and Mg ions, and GTP (Weisenberg, 1972; Olmsted & Borisy, 1975; Kobayashi & Shimizu, 1976). Among these factors, the effect of anion species upon both *in vitro* microtubule assembly (Sakai & Matsumoto, 1978) and the maintenance of the membrane excitability of squid giant axons called our particular attention.

The effect of K halides and K glutamate on the microtubule assembly efficiency was found to be in the order glutamate $\sim$ F > Cl > Br > I, in the previous study (Sakai & Matsumoto, 1978). The reduced viscosities attained to maxima were 1.40, 1.34, 0.92, and 0.27, respectively. This order is qualitatively in good agreement with the order of effect of these halides and glutamate on the survival time for excitability of the axon in perfusion experiments (Tasaki *et al.*, 1965). At the same time, the most favorable concentrations of KF and K glutamate for microtubule assembly, 100 and 300 mM, respectively (Sakai & Matsumoto, 1978), were found to agree with those for supporting the biggest sizes of action potentials or the lowest thresholds.

Colchicine would shift the equilibrium between microtubule assembly and disassembly in the axoplasm toward the disassembly by sequestering most of tubulin. It would work for slow disassembly of microtubules (Haga & Kurokawa, 1975), accounting for the gradual change of the action potential or threshold. The stronger effect of podophyllotoxin which is strongly competitive to colchicine (Wilson *et al.*, 1974) could be attributed to the higher reactivity of podophyllotoxin with tubulin than colchicine and its temperature independence. The same was true for vinblastine.

The effect of griseofulvin was similar to that of lumicolchicine. The former is known to bind to the high molecular weight protein (HMW)

associated with microtubules and to be an inhibitor of microtubule assembly (Roobol, Gull & Pogson, 1976; Weber, Wehland & Herzog, 1976). The requirement of a large excess amount of the reagent to induce microtubule disassembly may account for the slight effect of griseofulvin on the excitability of the giant axon. Furthermore, lumicolchicine is known to bind to some components of membranes from several non-neural cells (Stadler & Franke, 1974).

It has been well established that Ca ion is one of the most powerful agents to induce deterioration of the excitability (Tasaki *et al.*, 1967) and collapse of microtubules (for reviews, *see* Mohri, 1976, and Snyder & McIntosh, 1976). The former behavior of Ca ion was confirmed in this experiment. The latter was also confirmed in our previous study (Sakai & Matsumoto, 1978), revealing a good correlation. It should be noted that Ca ions also caused the resting potential to reduce rapidly. Since Ca ions would also bind to many sites other than tubulin, the interpretation cannot be given in so straightforward a manner as that given for colchicine and podophyllotoxin. Many experiments discussed the effect of internal Ca ions upon the Na current (Takahashi & Yoshii, 1978; Terekawa *et al.*, 1977; Begenisich & Lynch, 1974). The internal Ca ions would act at least on both microtubules in cytoskeleton and channel proteins in the plasma membrane.

The effects of SH reagents perfused internally upon action potential and resting potential were studied in detail by Baumgold, Matsumoto and Tasaki (1978). In this experiment, the effects of SH reagents depolymerizing microtubules (Kuriyama & Sakai, 1974) were classified into two groups: One includes NEM and diamide which are less specific for protein SH groups and the other DTNB and PCMB as more specific reagents. The latter group behaved like the action of podophyllotoxin. The biphasic behavior of threshold induced by the former group may indicate the existence of two binding sites for the SH reagents; one might be channel proteins, and the reagents might cause a translation of the steady-state Na inactivation along the voltage axis as observed with the effect of external Ca ions upon squid and *Myxicola* giant axons (Frankenhaeuser & Hodgkin, 1957; Schauf, 1975), and the other is tubulin. If we assume that reaction between DTNB and channel proteins would also cause a translation of the steady-state Na inactivation along the voltage axis but in an opposite direction to that produced by NEM or diamide, the ability to inhibit the excitability could be expected to be in the order of DTNB-PCMB group > NEM-diamide group, and this was the case for the experimental results. The reason dithiothreitol failed

to recover the excitability which had been partially suppressed by DTMB or PCMB could be due to the operation of perfusion. Some of the proteins which were indispensable for axonal excitability and modified by SH reagents would have been washed out together with some other soluble components.

In conclusion, close correlation was found between conditions for maintenance of the membrane excitability and for supporting microtubule assembly. It is probable that many microtubules are directly or indirectly associated with the plasma membrane. The most probable function of cytoskeletal microtubules close to the membrane is that they control the spatial interaction among functional or integral proteins regulating the ion permeability. From the physiological point of view, a strong coupling among functional proteins makes the threshold low and long-range interaction gives the big size of action potential since the increasing number of functional or excitable proteins can take part in one excitation.

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