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Axonal Microtubules Necessary for Generation of Sodium Current in Squid Giant Axons: I. Pharmacological Study on Sodium Current and Restoration of Sodium Current by Microtubule Proteins and 260K Protein

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Summary. Effects of the reagents suppressing or supporting axoplasmic microtubule assembly were studied on the Na ionic current of squid giant axons by perfusing the axon internally with the solution containing the reagent. Among the reagents suppressing the assembly, colchicine, vinblastine, podophyllotoxin, sulfhydryl reagents such as DTNB and NEM, and chaotropic anions such as iodide and bromide, were examined. These reagents reduced maximum Na conductance and shifted the voltage dependence of steady-state Na activation in a depolarizing direction along the voltage axis. They also made the voltage dependence less steep, but did not affect sodium inactivation appreciably. Effects on Na ionic current of reagents which support microtubule assembly (Taxol, DMSO, D_2O and temperature) were opposite the effects of those agents suppressing assembly. At the same time, we demonstrated that after Na currents were partially reduced, they could be restored by internally perfusing the axon with a solution containing microtubule proteins, 260K proteins and cAMP under conditions favorable for microtubule assembly. For full restoration, it was found that the following conditions were necessary: (1) The microenvironment within the axon is suitable for microtubule assembly. (2) Tubulins incorporated into microtubules are fully tyrosinated at their C-termini. (3) A peripheral protein having a molecular weight of 260,000 daltons (260K protein) is indispensable. These results suggest that axoplasmic microtubules and 260K proteins in the structure underlying the axolemma play a role in generating Na currents in squid giant axons.

Key Words Na current · axoplasmic microtubules · 260K proteins

Introduction

Axonal microtubules appear to play an important role in the maintenance and generation of membrane excitability in squid giant axons. Electron microscopic observation of the giant axon of squid shos that axoplasmic microtubules are only densely distributed near he inner surface of the axolemma and that they run almost parallel with the longitudinal axis of the axon forming networks with neurofilaments, thin filaments and cross-bridges (Metuzals & Tasaki, 1978; Endo, Sakai & Matsumoto, 1979; Hodge & Adelman, 1980; Matsumoto et al., 1982*a*, *b*). Evidence that these microtubules are important for excitation is physiological responses observed when the axon is exposed to conditions favorable or unfavorable for microtubule assembly. Internal application to the axon of reagents disrupting microtubules was found to bring about a decrease in the amplitude of action potentials, degradation of resting potentials and an increase in the current threshold (Matsumoto & Sakai, 1979*a*). Further, it was found that the reduced membrane excitability of the axon was partially restored by intracellularly perfusing it with a solution containing microtubule proteins under the conditions supporting the microtubule assembly (Matsumoto, Kobayashi & Sakai, 1979; Matsumoto et al., 1982*a*, *b*; Matsumoto & Sakai, 1979*b*).

Physiological role of axonal microtubules in the process of nerve excitation, however, is not yet altogether clear. Several previous studies showed that internal application to squid giant axons of reagents causing microtubule disassembly reduced the peak sodium current and affected the sodium activation without any appreciable effect on the fast sodium inactivation (Matsumoto et al., 1980, 1982*a*, *b*). The present and subsequent papers are prepared to clarify the physiological role of axonal microtubules and 260K proteins which were found only at the peripheral region inside the axolemma (Matsumoto et al., 1979, 1982*a*, *b*). In the present paper, we have studied by the voltage-clamp technique with compensation (Katz & Schwartz, 1974) the pharmacological effects both of reagents which suppress and those which support microtubule assembly upon the sodium, potassium and leak currents. We have also studied under voltage clamp the conditions necessary to restore the electrical excitability once it has been partially reduced. We

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have already succeeded in restoring the excitability of squid giant axons, judged from the size of action and resting potentials and of current threshold (Matsumoto et al., 1979, 1982*a*, *b*). The subsequent paper (Matsumoto, Ichikawa & Tasaki, 1984) will treat effects of colchicine upon the asymmetrical displacement current.

Materials and Methods

Materials

Giant axons of squid (*Doryteuthis bleekeri*) were used. For use in physiological experiments, the squid were collected in Sagami Bay, transported to the Electrotechnical Laboratory at Tsukuba where the physiological experiments were performed, and maintained in a small, circular and closed-system aquarium tank (Matsumoto, 1976; Matsumoto & Shimada, 1980). Although the aquarium system allowed the squid to survive in the tank for over 40 to 60 days, they were usually used up within 2 weeks of delivery. For biochemical extraction and purification of axoplasmic proteins, about one hundred of the squid collected in Sagima Bay were kept alive in a fish preserve at the Misaki Marine Biological Station. The squid thus preserved were used within one day after they were caught for dissection and collection of giant axons, bundles of fin nerve and optic ganglia.

Axon diameters were between 500 and 750 μm. The majority of the adherent tissues surrounding the axon was removed under a dissecting microscope.

Physiological Experiments

Experimental Procedures

Membrane currents of voltage-clamped axons bathed in artificial sea water (ASW) were measured as a function of time after the interal perfusion fluid was switched from the standard internal solution (SIS; *see* Table), which was composed of 380 mM K, 355 mM F and 25 mM HEPES (pH 7.2), to that containing a reagent supporting or suppressing microtubule assembly, for all the experiments except measurements of slow inactivation of the Na conductance. For the measurements of slow inactivation we used an internal perfusate whose ionic compositions were 380 mM K, 305 mM glutamate, 50 mM F and 25 mM HEPES (SIS-glutamate), pH being adjusted to be 7.2, to reduce the leakage current substantially and to allow holding potentials away from the resting potential for protracted periods of measurement time. For the restoration experiments, the membrane excitability was reduced by internally perfusing the squid giant axon with the SIS containing 15 to 20 mM colchicine. Then, the axon was internally perfused with the SIS again until the recovery reached a maximal level. Finally, the axon was internally perfused with the SIS containing microtubule proteins, 260K protein, tubulin-tyrosin ligase, the reagents activating the ligase such as ATP, Mg^{2+} and K^+ ions, tyrosine, GTP and/or cAMP (*see* Table). These proteins and reagents have been found to be effective and indispensable to restore membrane excitability (Matsumoto et al., 1979, 1982*b*; Matsumoto & Sakai, 1979*b*). The present experiments aim at elucidating functional roles of these proteins and reagents from the viewpoint of the Hodgkin-Huxley scheme (Hodgkin & Huxley, 1952).

The effect of a reagent upon the sodium current was studied by measuring peak sodium currents $I_{Na}^{peak}(V)$ as a function of membrane potential V. Then, the peak Na conductance $g_{Na}^{peak}(V)$ was obtained from the relation that $g_{Na}^{peak}(V)=I_{Na}^{peak}(V)/(V-V_{rev})$, where the reversal potential V_{rev} was experimentally determined with the $I_{Na}^{peak}-V$ curve. Voltage dependence of fast sodium inactivation was obtained with the double-pulse method, in which we did not put any time interval between pre- and test-pulses (Gillespie & Meves, 1980). The effect upon the potassium current was studied by measuring the late current $I_K(V)$. The steady state K conductance was obtained from the K current at the end of the voltage-clamp pulse (20 msec for the axon at 0 to 10 °C, and 9 msec at temperatures higher than 10 °C). Steady-state slow inactivation of the Na conductance in perfused squid axons was obtained as a function of membrane potential. The steady potassium conductance $g_K^\infty(V)$ was obtained by $I_K^\infty(V)/(V-V_K)$, where the potassium equilibrium potential V_K was approximated by calculating with use of the Nernst equation. The leak conductance g_L was determined from hyperpolarizing potential steps.

The holding potential was set at the resting potential of the axon for all the measurements except those of slow inactivation. Unless otherwise described, a test pulse was preceded by a prepulse of -100 mV with a duration of 30 msec in order to eliminate the effect of fast Na inactivation. We did not examine the effect upon slow Na inactivation (Adelman & Palti, 1969; Chandler & Meves, 1970; Rudy, 1978, 1981) of the reagents which affect microtubule assembly since the electrical excitability was reduced when the axon was held at hyperpolarizing potentials for a long period of time (longer than 10 sec), especially when the axon was internally perfused with a reagent suppressing microtubule assembly.

Intracellular Perfusion and Voltage Clamp

The axon was intracellularly perfused according to the method originally developed by Baker, Hodgkin and Shaw (1962) and modified by Narahashi, Anderson and Moore (1967). After the axon was put on transparent rubber laid on a Lucite® plate, the axoplasm was gently extruded using a rubber-coated roller while the axon was kept straight. The axon was then transferred to a Lucite chamber filled with ASW and was initially perfused with the SIS.

The chamber was similar to the one originally developed by Tasaki (1968) and improved for voltage-clamping by Armstrong, Bezanilla and Rojas (1973). The central and guard electrodes were made of platinized-platinum thin sheet which was pasted on a rectangular block of Lucite. The lengths of the central and guard electrodes were 6 and 3.5 mm, respectively. Temperature of the axon was kept constant within ± 0.05 °C at any temperature from 0 through 40 °C. The temperature control was principally performed by controlling temperature of the chamber itself within ± 0.01 °C. Internal and external electrodes, and the internal current electrode were similar to ones previously described (Matsumoto & Sakai, 1979*a*).

The voltage-clamp method with compensated feed-back was adopted to reduce the error caused by the resistance in series with the membrane (Katz & Schwartz, 1974). The voltage-clamp system we used was, in principle, similar to the one used by Bezanilla and Armstrong (1977). A block diagram of the experimental setup is shown in Fig. 1. The setup consisted of two main sections: an analog section and a digital section. Communication between both sections was by way of optical isolators (Yokogawa-Hewlett Packard Co., type HCPL 2630) of 2 bits in size and 50 nsec in settling time, in order to prevent ground loops between the two sections. The analog section

contained a sample-and-hold amplifier (Analog Devices Co., type THC 300) with 0.25 μsec acquisition time (error within ±0.01%), a 12 bit analog-to-digital converter (ADC) (Micro Networks Co., model MN 5245) with a 1 μsec conversion time, and a 12 bit digital-to-analog converter (DAC) (Datel Co., model HF 12) with 50 nsec conversion time. The digital section contained a personal microcomputer system (Sharp Co., model MZ-80B), a timing-control circuit mainly composed of a microprocessor unit (Sharp Co., type Z-80A) and a crystal clock with the basic oscillation frequency of 8.000 MHz, and a 16K word buffer memory (Toshiba Co., type TMM 5516). The timing-control circuit was necessary to adjust the timing between the initiation time for DAC and the initiation time for data acquisition at the ADC correctly (within 10 nsec). We put the buffer memory outside the host microcomputer system. Analog data of membrane currents were first stored in the buffer memory directly, and then transferred to the memory of the host microcomputer under computer control for data processing. The data thus processed were stored on mini-floppy disks. Our voltage-clamp system thus prepared could produce clamp voltages within 0.1% of the command voltage and the system was operated with a linearity error of ±0.025%.

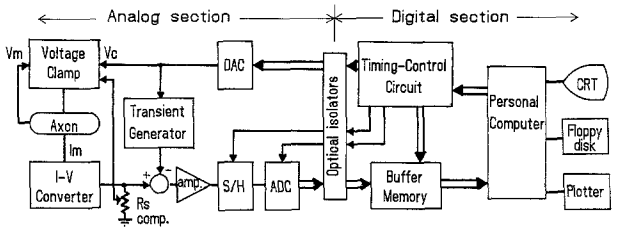

Fig. 1. Schematic diagram of the voltage clamp system used in the experiments

Solutions, Reagents and Protein Preparation

Solutions

The compositions of both internal and external solutions used in this experiment are listed in the Table. Both internal and external solutions were perfused. The flow rate of the internal perfusion solution was usually 10 to 20 μl/min, but sometimes the flow was almost stopped at the rate of 0.5 to 5 μl/min to examine the effect of a reagent in the solution upon the membrane excitability under the condition of almost no flow. The flow rate of the external perfusion solution was usually 0.5 to 1 ml/min.

Reagents

The reagents used in this study are divided into two categories; the reagents favorable and unfavorable to support microtubule assembly. The reagents against microtubule assembly are colchicine, podophyllotoxin, vinblastine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and N-ethylmaleimide (NEM). The reagents for supporting microtubule assembly are Taxol, dimethyl

Table 1. Components of the internal and external solutions

A) Internal solution[a] (the unit is mM unless otherwise specified)

Name	K	Mg	F	Glutamate	Cl	I	Br	HEPES[b]	Porcine brain C_3S	Squid brain C_2S (mg/ml)	Squid axoplasmic C_2S	Tubulin tyrosine ligase	260K protein (mg/ml)	Tyrosine	GTP	cAMP	ATP
Standard internal solution (SIS)	380		355					25									
SIS-glutamate	380		50	305				25									
305KI−50KF	380		50			305		25									
305KBr−50KF	380		50				305	25									
Squid brain-GTP-Mg	380	3	355		6			25		0.5–1					0.5		
Squid brain-260K	380	3	355		6			25		1			0.05–0.3		0.5		
Squid brain-260K-cAMP	380	3	355		6			25		1–5			0.1–0.3		0.5	0.005	
Squid axoplasmic C_2S-260K-cAMP	380	3	355		6			25			1 mg/ml		0.15		0.5	0.005	
Porcine brain-260K	380	3	355		6			25	1 mg/ml				0.12		0.5		
Porcine brain-ligase	380	3	355		6			25	1 mg/ml			3 U/ml		0.03	0.5		0.5–1
Porcine brain-260K-ligase	380	3	355		6			25	1 mg/ml			3 U/ml	0.05–0.15	0.03–0.06			

[a] HEPES and Tris were used as buffers. pH was adjusted to 7.3 for HEPES and 8.2 for Tris, respectively

[b] Glycerol was used to adjust osmolality to 980 mosmol/kg

B) External solution[a] (mM)

Name	K	Ca	Na	Tris[b]	Cl
Artificial sea water (ASW)	10	44	400	10	504

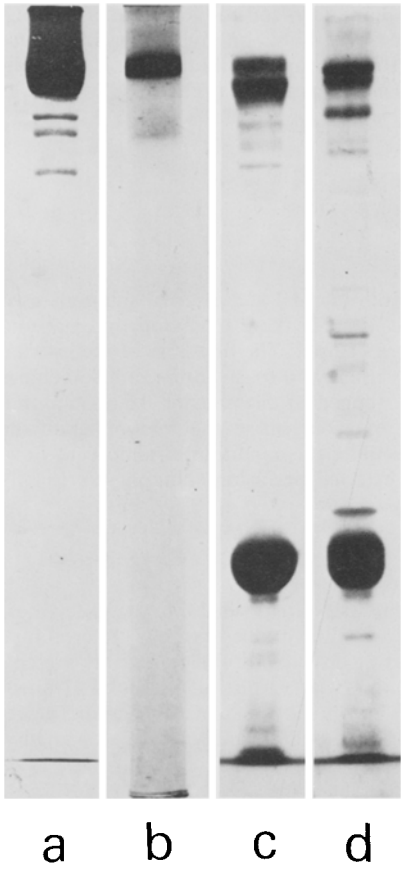

Fig. 2. Electrophoretic patterns of 260K protein and microtubule proteins from porcine and squid brains. Lanes *a* and *b*, Con A-Sepharose fraction for 260 K protein; *c*, porcine brain microtubule proteins (C_3S); *d*, squid brain microtubule proteins (C_2S). Electrophoresis was done according to Laemmli (1970) using 7.5% polyacrylamide gels for lanes *a*, *c* and *d*, and after Ohtsubo et al. (1975) using 4% polyacrylamide gel containing 8 M urea for lane *b*

sulfoxide (DMSO) and an appropriate concentration of deuterium oxide (D_2O). Taxol was a kind gift from the Natural Products Branch, Division of Cancer Treatment, NCI. Five millimolar Taxol was dissolved into DMSO as a stock solution and kept in a freezer of −80 °C until use. Other reagents except for podophyllotoxin and D_2O were purchased from Sigma Chemical Co. (St. Louis, Mo.). Podophyllotoxin and D_2O were obtained from Aldrich Chemical Co. (Milwaukee, Wis.) and Merck Chemicals (Darmstadt, Germany), respectively. The reagents were used without any further purification.

Protein Preparation

For the restoration experiments we extracted and purified the following proteins and enzymes from squid giant axons, bundles of squid fin nerve, optic ganglia of squid, porcine brains and/or rabbit skeletal muscles.

Axoplasmic 260K Protein. A high molecular weight protein (260K protein) was extracted from squid giant axons according to Sakai and Matsumoto (1978) with slight modifications. The yield of 260K protein (Con A-sepharose fraction) was 0.072 mg per squid. Figure 2 illustrates the electrophoretic pattern of 260K protein in the presence of SDS (gel *a*). Electrophoresis was done according to Laemmli (1970) using 7.5% polyacrylamide gel, or according to the method of Ohtsubo et al. (1975) using 4% polyacrylamide gel containing 8 M urea which was a modification of the method of Weber and Osborn (1969). On gel *a*, 260K protein exhibited a broad band near the top of the separation gel presumably because of partial aggregation caused by extensive condensation of proteins in the spacer gel, but it became a distinct band (gel *b*) when the electrophoresis was performed after Ohtsubo et al. (1975) which omitted the spacer gel. Densitometric analysis of the gel revealed that the 260K protein thus prepared (Con A-Sepharose fraction) was about 80% pure. The purification of 260K protein was reported elsewhere (Murofushi et al., 1983). The 260K protein had been called "300K protein" in our previous papers (Matsumoto et al., 1979, 1982*a*, *b*). We renamed it since its molecular weight was found to be 260,000, as judged from the analysis of SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970) (Matsumoto et al., 1982*b*; Murofushi et al., 1983).

Microtubule Proteins. Porcine brain microtubule proteins (C_3S) were prepared from porcine brains by three cycles of temperature-dependent polymerization and depolymerization according to Shelanski, Gaskin and Cantor (1973) with slight modifications (Kuriyama, 1975). Microtubule pellets formed by the third cycle of polymerization were homogenized with 0.3 M potassium glutamate − 10 mM MES (pH 6.9) − 1 mM EGTA − 0.5 mM $MgCl_2$ − 0.5 mM GTP and kept at −80 °C. Electrophoresis of this protein fraction on an SDS-polyacrylamide gel is shown in Fig. 2(*c*). Squid brain microtubule proteins (C_2S) were prepared from the optic ganglia in the same manner, except for 2 cycles at 25 and 0 °C (Sakai & Matsumoto, 1978). This fraction is shown in Fig. 2(*d*). Axoplasmic microtubule proteins (C_2S) were prepared from axoplasm extruded from squid giant axons (Sakai & Matsumoto, 1978) in the same manner as those for squid brain C_2S.

Tubulin-Tyrosine Ligase. This enzyme was purified from porcine brains according to Murofushi (1980).

Neurofilaments. Neurofilaments were prepared from porcine brains by the method of Runge, Schlaepfer and Williams (1981).

Actin. Skeletal muscle actin of rabbit was prepared after Spudich and Watt (1971). Porcine brain actin was purified by chromatography on DEAE-cellulose and Sepharose-sebacic acid hydrazide-ATP followed by polymerization and depolymerization.

The amount of protein was determined according to Lowry et al. (1951).

Results

Reagents Suppressing Microtubule Assembly

Anti-Mitotic Reagents (Colchicine, Podophyllotoxin and Vinblastine)

Colchicine affected the Na current appreciably but had little effect on the K and leak currents. Figure 3(*A*) shows one of the results where the axon was internally perfused with the SIS containing

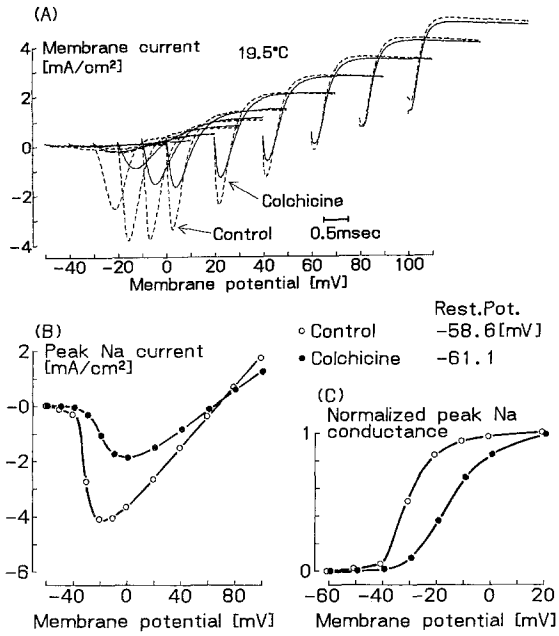

Fig. 3. Effect of colchicine upon the ionic currents after the axon was internally perfused with the SIS containing 20 mM colchicine for 16 min at the flow rate of about 2 µl/min at 19.5° C. (*A*) Membrane currents before (dotted lines) and after the colchicine treatment (solid lines). The clamp potentials are −50, −40, −30, −20, −10, 0, 20, 40, 60, 80 and 100 mV, respectively. The pulse duration was 5 msec each. The holding potential was set at the resting potentials of −58.6 mV for the control and of −61.1 mV for the colchicine-treated axon, respectively. A prepulse of membrane potetial −100 mV with the duration of 30 msec preceded the test pulse. (*B*) Voltage dependence of the peak Na currents for the axon treated and untreated with colchicine, obtained from the data in the above figure (*A*). (*C*) Voltage dependence of the peak Na conductances normalized by the average one obtained at 20 to 80 mV for the axon treated and untreated with colchicine

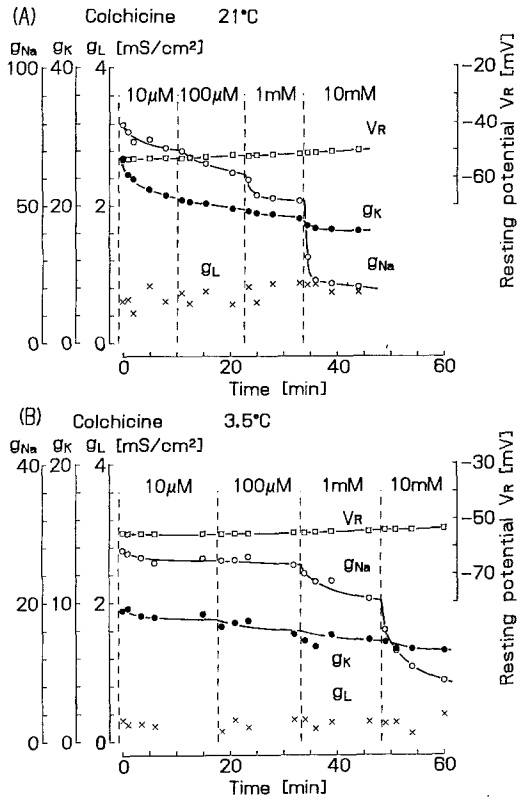

Fig. 4. Sodium, potassium and leak conductances, and the resting potential for the colchicine-treated axon as a function of perfusion time at 21 °C (*A*) and at 3.5 °C (*B*), respectively. The axon was internally perfused with the SIS containing 10 µM, 100 µM, 1 mM and 10 mM colchicine, successively, under the usual flow rate of 10 to 20 µl/min

20 mM colchicine at 19.5 °C, under almost no flow condition (at the flow rate of about 2 µl/min). It was found that colchicine reduced the maximum Na conductance, and at the same time, affected the sodium activation (Fig. 3*B* and *C*). Effect of colchicine upon the Na activation will be studied in more detail by measuring asymmetrical displacement current in our following paper. Colchicine also did not produce any effect on reversal potentials (Fig. 3*A*) and sodium inactivation (*see below*).

On the other hand, when the axon was internally perfused with the SIS-containing colchicine at the usual flow rate of 10 to 20 µl/min rather than almost no flow conditions, colchicine affected more appreciably not only the sodium but also potassium currents in a concentration-dependent manner. Figure 4 illustrates dependence of the membrane conductances and resting potentials on colchicine concentration at 21 °C (*A*) and 3.5 °C (*B*), respectively, when the axon was intracellularly perfused with SIS containing 10 µM, 100 µM, 1 mM and 10 mM colchicine in succession at the usual perfusion flow rate. In the example at 21 °C (Fig. 4*A*), even 10 µM colchicine was effective in reducing the Na conductance, as had been found previously (Matsumoto et al., 1982*a*, *b*), but at 3.5 °C (Fig. 4*B*), 1 mM colchicine was necessary. In both examples, the effect of colchicine on the Na conductance was much greater than that on the K and leak conductances. The effect of a specific concentration of colchicine on the Na conductance saturated within a specific time (Fig. 4).

The effect of colchicine upon the sodium activation was also concentration dependent. The 1 mM colchicine shifted the normalized peak Na conductance versus voltage curve along the voltage axis in the positive direction by 2 to 5 mV at 19.8 to 21 °C and by 4 to 6 mV at 3.3 to 5.5 °C, respectively. Ten to 20 mM colchicine not only shifted the curve along the voltage axis by 16 to 33 mV

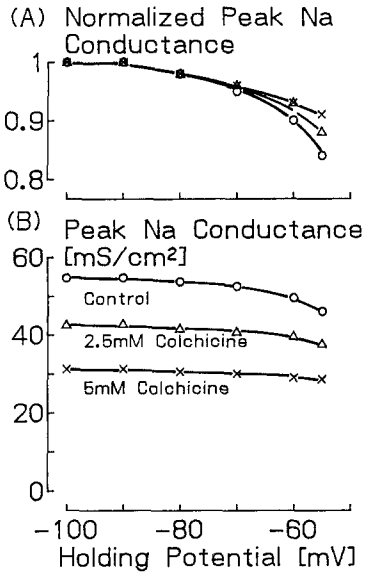

Fig. 5. Membrane potential dependence of steady-state slow inactivation of the Na conductance. An axon immersed in ASW was internally perfused with 25 TEA-SIS-glutamate (control, o), with 25 TEA-SIS-glutamate containing 2.5 mM colchicine (record ×) and 5 mM colchicine (record △), in succession. After the axon was held at a specific membrane potential for 10 sec, a prepulse of −100 mV with a duration of 30 msec preceded a test pulse in order to eliminate the effect of fast inactivation. 16 °C. (*A*) Normalized peak Na conductance *vs.* holding potential curves for the control and colchicine-treated axon, where the peak Na conductance was normalized at the value of the peak Na conductance at the holding potential of −100 mV. (*B*) Peak Na conductance *vs.* holding potential curves for the control and colchicine-treated axon

at 19.8 to 21 °C and by 11 to 18 mV at 3.3 to 5.5 °C, respectively, but also made the voltage dependence less steep (Fig. 3*C*).

As previously observed (Matsumoto et al., 1980, 1982*a*, *b*), colchicine did not give any detectable effect on the steady-state fast sodium inactivation unless its concentration exceeded 10 mM even with normal perfusion flow. It can be seen in Fig. 2 of our subsequent paper that the time course of fast sodium inactivation after 2.5 mM colchicine treatment is in good agreement with that before colchicine treatment (control). However, the 10 mM colchicine shifted the steady Na inactivation, $h_\infty(V)$, along the voltage axis in the negative direction by 5 to 7 mV at 20 to 22 °C. Colchicine also did not produce any appreciable effect on the steady-state slow sodium inactivation. A typical experimental result of its voltage dependence is illustrated in Fig. 5, where the axon immersed in ASW was internally perfused with the SIS-glutamate containing 25 mM tetraethylammonium chloride (25 TEA-SIS-glutamate), the 25 TEA-SIS-glutamate containing 2.5 and 5 mM colchicine, successively. The steady-state slow inactivation *vs.* voltage curves were found to be similar for control and colchicine-treated axons, where the colchicine concentration examined in the present experiment was below 5 mM and the holding potentials were between −50 and −100 mV. The curves were found to be in reasonable agreement with those obtained by others (Adelman & Palti, 1969; Chandler & Meves, 1970; Rudy, 1978, 1981).

The reversal potential was unaffected for axons treated with colchicine at concentrations less than 20 mM with slow perfusion flow rates (0.5 to 2 µl/min (Fig. 3*A*). Ten to 20 mM colchicine at the usual flow rate of 10 to 20 µl/min reduced the reversal potential by 1 to 7 mV, as previously observed (Matsumoto et al., 1982; 1982*b*).

Compared to colchicine, vinblastine and podophyllotoxin gave similar but stronger effects upon the Na, K and leak currents. It was found that 2 mM vinblastine at 16 to 20 °C corresponded to 10 to 20 mM colchicine at 20 °C. At low temperatures of 3 to 6 °C, it was also found that podophyllotoxin was much more effective than colchicine in reducing the peak Na conductance; that is, 1 mM podophyllotoxin reduced the peak conductance by about 50% as compared with the control while 1 mM colchicine reduced Na conductance by less than 25% at these temperatures. The difference of the effect would be larger if we consider the poor solubility of podophyllotoxin in the SIS.

Sulfhydryl Reagents (DTNB and NEM)

It is known that sulfhydryl reagents reduce the peak sodium currents while Na inactivation remains normal (Oxford, Wu & Narahashi, 1978). These results were confirmed in the present experiments. Further, the curve of the normalized peak Na conductance versus membrane potential was shifted in the positive direction along the voltage axis and, at the same time, the voltage dependence became less steep with time after the axon was internally perfused with the SIS containing 5 mM DTNB or 1 to 3 mM NEM continuously. The reversal potential was unchanged during 55 min of perfusion with 5 mM DTNB at 16.6 °C. The 5 mM DTNB gave a weak effect upon the K conductance but an appreciable effect on the K activation. The effect of internal perfusion with 1 to 3 mM NEM at 16.5 °C was found to be similar to that of DTNB.

Anions (F^-, Glutamate, Cl^-, Br^-, I^-)

It had been reported (Sakai & Matsumoto, 1978) that the order of anions for supporting microtu-

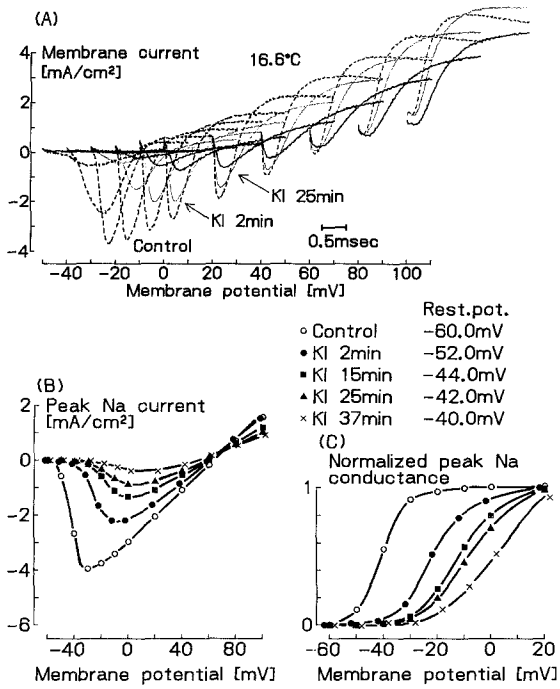

Fig. 6. Effect of chaotropic anion I^- upon the membrane currents for the axon internally perfused with the SIS (control) at the onset of perfusion and then with the 305KI–50KF for 37 min continuously under the usual flow rate of 10 to 20 µl/min at 16.6 °C. (*A*) Voltage dependence of the membrane currents for the control axon (broken lines) and for the axon perfused with the 305KI–50KF for 2 min (thin solid lines) and for 25 min (heavy solid lines). Membrane potentials applied were −50, −40, −30, −20, −10, 0, 20, 40, 60, 80 and 100 mV, respectively. (*B*) Voltage dependence of the peak Na current for the control axon (○) and for the axon perfused with the 305KI–50KF for 2 min (●), 15 min (■), 25 min (▲) and 37 min (×), respectively. (*C*) Voltage dependence of normalized peak Na conductance for the control axon (○) and for the axon perfused with 305KI–50KF for 2 min (●), 15 min (■), 25 min (▲) and 37 min (×), respectively

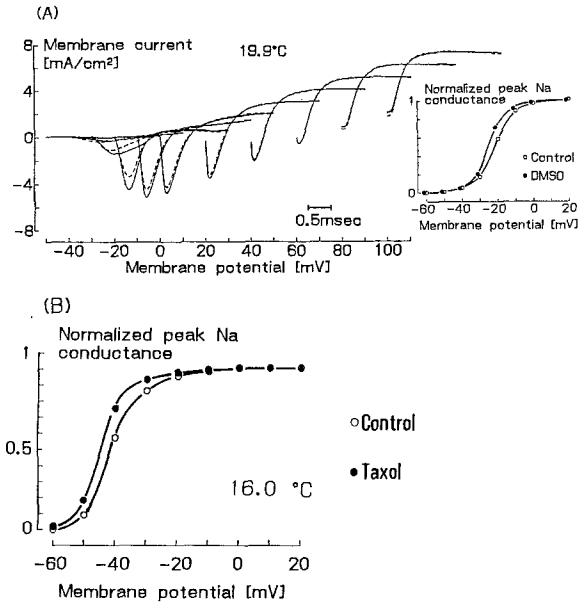

Fig. 7. (*A*) Effect of DMSO on the membrane currents for the axon internally perfused with the SIS (control) at the onset of perfusion and then with the SIS containing 1 mM DMSO for 30 min under slow flow rate of less than 5 µl/min at 19.9 °C. Voltage dependence of the membrane current for the control axon (dotted lines) and for the DMSO-treated axon (solid lines). Membrane potentials applied were −50, −40, −30, −20, −10, 0, 20, 40, 60, 80 and 100 mV, respectively. *Inset:* Voltage dependence of normalized peak Na conductance for the control (○) and DMSO-treated (●) axon. (*B*) Voltage dependence of normalized peak Na conductance for the control (○) and Taxol-treated (●) axon. The axon was internally perfused with the SIS (control) and then with the SIS containing 20 µM Taxol (and 50 µM DMSO) for 65 min under slow flow rate of 2 µl/min at 16.0 °C

bule assembly was in good agreement with the one favorable for maintaining the electrical excitability of squid giant axons (Tasaki, Singer & Takenaka, 1965). Now, the problem was why chaotropic anions such as I^- and Br^- destroyed the excitability. The effect of internal perfusion at 16.6 °C with 305KI–50KF was examined (Fig. 6). The peak Na conductance decreased with the perfusion time (Fig. 6). At the same time, the voltage dependence of the normalized peak Na conductance was appreciably shifted in the positive direction along the voltage axis and became less steep with perfusion time, while the reversal potential was unchanged (Fig. 6). The effect of internal perfusion with 305KI–50KF upon the Na current was found far stronger than that upon the K current (Fig. 6). The effect of internal perfusion with 305KBr–50KF was weaker than but similar to the effect of 305KI–50KF.

Reagents Supporting Microtubule Assembly

DMSO

Dimethyl sulfoxide (0.1 to 1 mM) increased the peak Na current at 16 to 21 °C (Fig. 7*A*). The Na current, when the axon was perfused with the SIS containing 1 mM DMSO for 30 min under the slow flow rate of less than 5 µl/min, increased 1.12 times as compared with that of the control, while the K current remained unchanged (Fig. 7*A*). The voltage dependence of the normalized peak Na conductance was shifted to the negative direction along the voltage axis, and became steeper as compared with that of the control (*see* inset of Fig. 7*A*). The reversal potential was unchanged before and after the DMSO treatment. Resting potential changed from −54.1 mV for the control axon to −57.5 mV for the DMSO-treated axon for 30 min.

Taxol

Taxol is an antimitotic drug derived from the western yew plant and has been shown to stimulate dramatically the polymerization of cytoplasmic microtubules *in vitro* (Schiff, Fant & Horwitz, 1979; Vallee, 1982) and *in vivo* (Schiff & Horwitz, 1980). Effects of 20 μM Taxol on Na and K currents were found to be very similar to those of 1 mM DMSO (Fig. 7*B*); the maximum peak Na conductance, when the axon was internally perfused with SIS containing 20 μM Taxol (and at the same time 50 μM DMSO) for 65 min under the slow flow rate of 2 μl/min, increased 1.1 times as compared with that of the control axon, while the K current remained almost unchanged. At the same time, the voltage dependence of the normalized peak Na conductance was shifted to the negative direction along the voltage axis, and became steeper than that of the control axon. Resting potential changed from −61 mV for the control axon to −65.7 mV for the Taxol-treated axon for 65 min.

D_2O

It is well known that substitution of D_2O for H_2O in the external solution alters conductance parameters ($\bar{g}_{Na}$, $\bar{g}_K$ and $\bar{g}_L$) and rate constants (α_m, β_m, α_h, α_n and β_n) in the Hodgkin-Huxley equations (Hodgkin & Huxley, 1952) through a single mechanism (Conti & Palmieri, 1968). This conclusion was obtained from observations of membrane currents about 3 min after the normal ASW was switched to ASW containing D_2O, since exchange of D_2O between external medium and axoplasmic side was completed almost immediately (15 sec) after the substitution. We have confirmed the above results of Conti and Palmieri (1968). Further, we observed change of membrane currents for 60 min after the normal ASW surrounding the intact axon was switched to the D_2O-ASW. The peak Na currents increased or decreased, compared with those obtained 1 to 3 min after the substitution, depending on the D_2O concentration in the D_2O-ASW (*see* inset of Fig. 8). Figure 8 shows a change of the peak Na current thus obtained at 50 min after the substitution. Note that the maximum increase of the peak Na current was obtained when the axon was immersed in the 45% D_2O-ASW, and that the peak Na current for the axon bathed in the 100% D_2O-ASW was monotonically reduced with time after the substitution of D_2O for H_2O. Since reversal potentials determined by the peak Na current versus membrane potential curve remained unchanged before and after the substitution, the

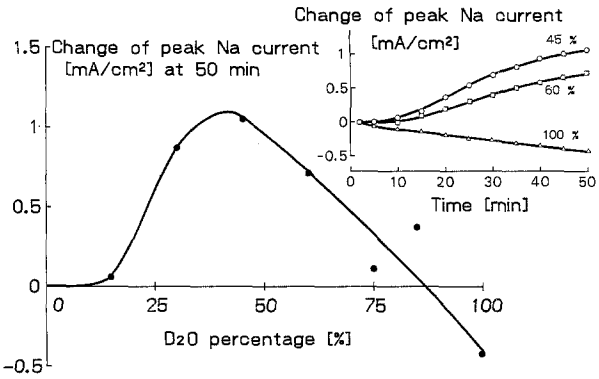

Fig. 8. Effect of D_2O concentration in ASW upon change of the peak Na current when the intact axon was bathed initially in normal ASW and then in a mixture of normal (100% H_2O-) ASW and 100% D_2O-ASW at 20 °C. The peak Na current gradually changed during 50 min after switching the external medium from normal ASW to the D_2O containing ASW, as compared with the one at 1 to 3 min (*see* inset). Changes of the peak Na current at 50 min as compared with the one at 1 to 3 min were illustrated as a function of D_2O concentration

change of the peak Na current exactly corresponds to that of the peak Na conductance. At the same time, the voltage dependence of the normalized peak Na conductance for the axons immersed in the 15 to 75% D_2O-ASW was shifted in the negative direction along the voltage axis and became more voltage dependent with time, as compared with that obtained at 1 to 3 min after the substitution. An opposite effect on the voltage dependence of the peak Na conductance was observed for axons bathed in 85 to 100% D_2O-ASW. On the other hand, the steady-state K currents were not increased during the observation period of 60 min, as compared with those obtained 1 to 3 min after the substitution.

Temperature

The effect of temperature on the various parameters in the Hodgkin-Huxley equations (Hodgkin & Huxley, 1952) has been extensively studied by many researchers. However, the studies have concentrated on clarifying the characteristics of the parameters below 15 °C (Hodgkin & Huxley, 1952; Schauf, 1973; Bezanilla & Taylor, 1978; Kimura & Meves, 1978; Matteson & Armstrong, 1982). Our interest is in the characteristics of the parameters around 25 °C since the optimal temperature for assembly of axoplasmic microtubules in squid giant axons was found to be around 25 °C (Sakai & Matsumoto, 1978). It was also found that the maximum peak Na current was obtained when the axon was at temperatures around 21 to 25 °C, and at the same time, that the curve of the normalized

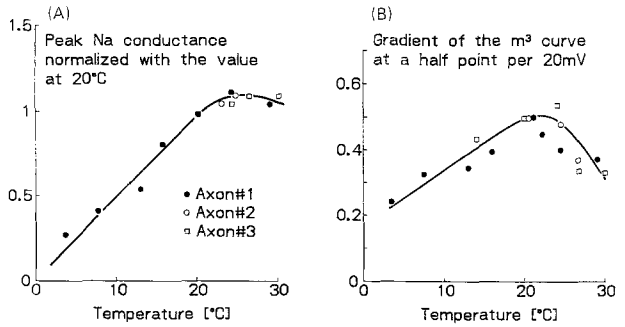

Fig. 9. Effect of temperature on peak Na conductance (*A*) and the gradient of the m^3 curve at a half point (*B*). The gradient was obtained by measuring the gradient for the curve of normalized peak Na conductance versus membrane potential at its half point

peak Na conductance versus membrane potential (that is, the m^3 curve) changes most steeply at temperatures of 21 to 25 °C. These results can be seen more clearly in Fig. 9, which shows the temperature dependence of the peak Na conductance, obtained for 3 axons and normalized with the value at 20.0 °C for each axon (*A*) and of the gradient of the m^3 curve at its half point (*B*) for the same 3 axons. It should be noted that Q_{10} of the peak Na conductance below 20 °C was 2.0 which was in reasonable agreement with the range of 1.9 to 3.9 reported by Kimura and Meves (1979) and the value of 2.2 by Matteson and Armstrong (1982). Reversal potentials were unchanged for temperatures from 3 through 30 °C.

Restoration of Electrical Excitability

The ability of microtubule proteins obtained from squid and porcine brains to restore Na current were separately examined mainly because cytoplasmic tubulins from squid nerve fully retained C-terminal tyrosine but those from porcine brains did not (Kobayashi & Matsumoto, 1982). It was previously found that cytoplasmic tubulins from porcine brains retained C-terminal tyrosine at 30 to 60% of the sites available for tyrosination (Kobayashi & Matsumoto, 1982).

Restoration by Internal Application of Squid Brain Microtubule Proteins

First, we investigated the effect of the solution "squid brain-GTP-Mg" (*see* Table) on the restoration of excitability in 8 axons at 20 to 22 °C. The concentration of the microtubule protein in SIS was 0.5 to 1 mg/ml. Axons were kept at 20 to 22 °C throughout the experiments since the optimal temperature was around 25 °C for assembling microtubules in squid axoplasm (Sakai & Matsumoto, 1978). It was found that 0.5 to 1 mg/ml squid brain C_2S, 0.5 mM GTP and 3 mM $MgCl_2$ dissolved in SIS (squid brain-GTP-Mg) was ineffective for restoration. Further, it was found that omission of GTP was totally ineffective in the restoration for 3 axons examined. It is well known that 0.1 to 0.5 mM Mg^{2+} ions and 0.5 mM GTP are necessary for microtubule assembly (Weisenberg, Borisy & Taylor, 1968; Olmsted & Borisy, 1975).

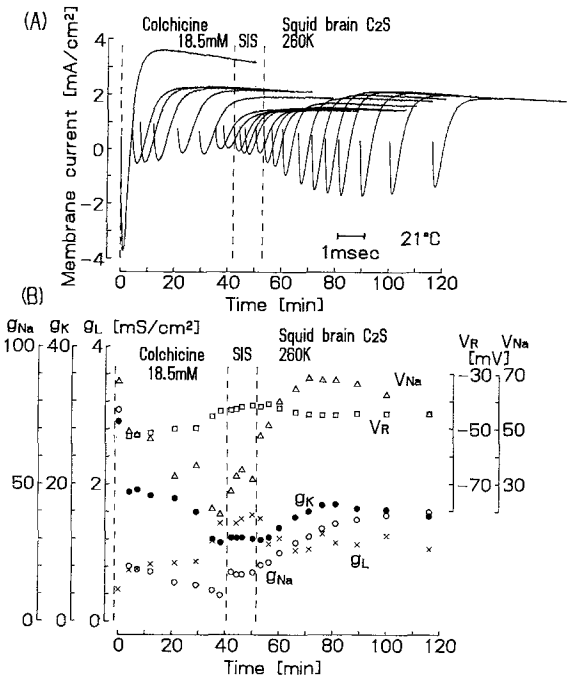

Fig. 10. Effect of the "squid brain-260K" on the restoration at 21.0 °C. The "squid brain-260K" was composed of 1 mg/ml squid brain C_2S, 0.5 mM GTP, 3 mM $MgCl_2$, 0.12 mg/ml 260K protein and the SIS (380 mM K, 355 mM F, 25 mM HEPES; pH 7.3). The squid giant axon immersed in ASW was intracellularly perfused with the SIS (until at 0 min), the SIS containing 18.5 mM colchicine (from 0 through 41 min), the SIS again (from 41 through 51.5 min) and the squid brain-260K (from 51.5 through 115 min), successively. (*A*) Changes of the membrane currents as a function of the perfusion time. The axon was voltage-clamped at a membrane potential of 20 mV after preconditioned with a pulse of −100 mV for 30 msec. (*B*) Changes of the peak Na conductance g_{Na} (○), steady K conductance g_K (●), leak conductance g_L (×), resting potential V_R (□) and reversal potential V_{Na} (△), as a function of the perfusion time, respectively

The "squid brain-260K" (1 mg/ml squid brain C_2S, 0.5 mM GTP, 3 mM $MgCl_2$ and 0.005 to 0.3 mg/ml 260K protein dissolved in SIS; *see* Table) restored the electrical excitabilities for 8 axons examined, as illustrated in Fig. 10 as one of the examples: (1) The Na, K and leak currents were more or less restored. (2) The Na current was much more restored than was the K current. (3) The de-

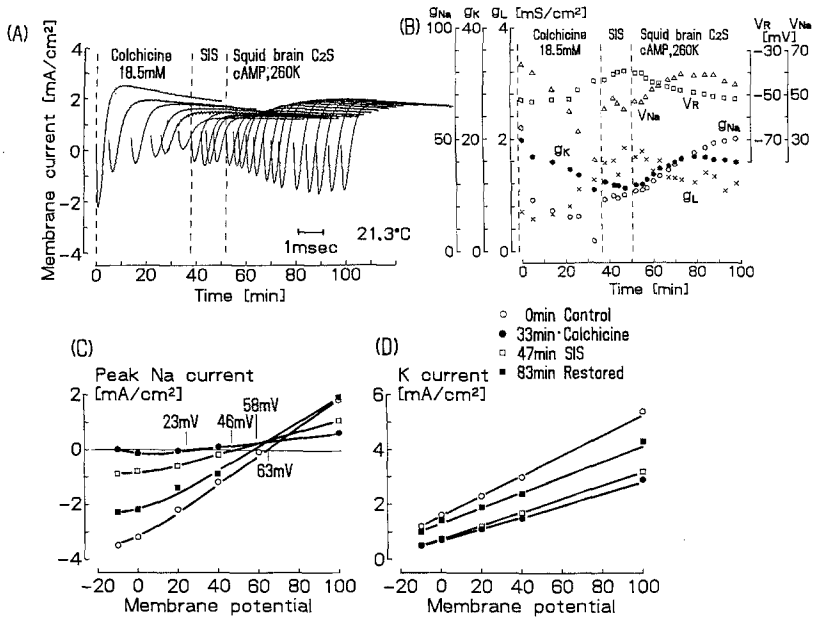

Fig. 11. Effect of the "squid brain-260K-cAMP" on the restoration at 21.3 °C. The "squid brain-260K-cAMP" was composed of 1 mg/ml squid brain C_2S, 0.5 mM GTP, 3 mM $MgCl_2$, 0.1 mg/ml 260K protein, 5 μM cAMP and the SIS (380 mM K, 355 mM F, 25 mM HEPES; pH 7.3). The squid giant axon immersed in ASW was intracellularly perfused with the SIS (until at 0 min), the SIS containing 18.5 mM colchicine (from 0 through 36.5 min), the SIS again (from 36.5 through 50.5 min) and the "squid brain-260K-cAMP" (from 50.5 through 97 min), successively. (*A*) Changes of the membrane currents as a function of the perfusion time. The axon was voltage-clamped at membrane potential of 20 mV after preconditioned with a pulse of −100 mV for 30 msec. The holding potential was kept at the resting potential. (*B*) Changes of the peak Na conductance g_{Na} (○), steady K conductance g_K (●), leak conductance g_L (×), resting potential V_R (□) and reversal potential V_{Na} (△), as a function of the perfusion time, respectively. (*C*) Voltage dependence of the peak Na current. (*D*) Voltage dependence of the K current. Measurements in both cases (*C*) and (*D*) were performed when the axon was internally perfused with the SIS (○) at 0 min, with the SIS containing 18.5 mM colchicine (●) at 33 min, with the SIS again (□) at 47 min, and with the "squid brain-260K-cAMP" (■) at 83 min, respectively. The K current was obtained as the membrane current at 5 msec from application of the voltage-clamp pulse

gree of the restoration was larger with increasing 260K protein concentration. However, the restoration attained a plateau at a 260K protein concentration above 0.12 mg/ml. (4) The time necessary for the maximal restoration was 40 to 50 min after switching of the internal solution to the "squid brain-260K." (5) Reversal potentials also recovered but gradually decreased with time. For example, in the axon shown in Fig. 10, reversal potentials at 0 min (for the control), 38 min (end of the perfusion with colchicine), 50 min (end of the second perfusion with SIS), 70 min and 115 min (saturation of the recovery of the Na conductance) were 68, 18, 32, 69 and 55 mV, respectively.

Effects of the "squid brain-260K-cAMP" (1 to 5 mg/ml squid brain C_2S, 3 mM $MgCl_2$, 0.5 mM GTP, 0.1 to 0.3 mg/ml 260 K protein and 5 μM cAMP dissolved in SIS; *see* Table) were investigated for 7 axons at 20 to 22 °C. Generally the degree of the restoration was largest among the combinations of reagents examined in the present experiments. In this case, the Na current was again much more restored than the K current, and also, the time necessary for the maximal restoration was 40 to 60 min after switching of the internal solution into the "squid brain-260K-cAMP." Further, reversal potentials also recovered. For example, in the axon shown in Fig. 11, reversal potentials at 0 min (for the control), 33 min (end of the colchicine treatment), 46.5 min (end of the second perfusion with the SIS) and 87.5 min (at the best recovery) were 63, 23, 46 and 58 mV, respectively. Resting potentials also completely recovered to the original levels of the control axon, as seen in Fig. 11 *B*. It should be noted that the K activation was also recovered with the K conductance. Using the "squid brain-260K-cAMP," changes in the concentration of microtubule proteins resulted in no appreciable difference in restoration for concentrations 1 and 5 mg/ml squid brain C_2S. We also investigated the effect of the "squid brain-260K-cAMP-ligase" composed of the "squid brain-260K-cAMP," 3 U/ml tubulin-tyrosine ligase, 50 μM tyrosine and 1 mM ATP, upon the restoration for 4 axons, and found that there was no further improvement in the restoration. This was performed to clarify whether microtubules completely composed of tyrosinated tubulins or tyrosinated

tubulin-dimers could contribute to the restoration (*see also* Restoration by Porcine Brain Microtubule Proteins).

Since the purified squid microtubule proteins (C_2S) contained detectable amounts of the components, the molecular weights of which corresponded to neurofilament triplet and actin (Metuzals & Tasaki, 1978; Pant et al., 1978; Sakai & Matsumoto, 1978; Zackroff & Goldman, 1980: *see also* Fig. 2*d*), we examined "squid brain-260K-cAMP" containing 0.1 mg/ml neurofilaments or 0.1 mg/ml actin with 0.1 mM ATP upon the restoration. The level of the restorations by the "squid brain-260K-cAMP" containing neurofilaments or actin were found to be almost identical to those by the "squid brain-260K-cAMP."

The restoration of the excitability by internal application of the "squid axoplasmic C_2S-260K-cAMP" (1 mg/ml squid brain C_2S, 3 mM $MgCl_2$, 0.5 mM GTP and 0.15 mg/ml 260K protein dissolved in the SIS; *see* Table) was examined for 2 axons, with results similar to those obtained by the "squid brain C_2S-260K-cAMP."

Restoration by Internal Application of Porcine Brain Microtubule Proteins

The "porcine brain-ligase" (Table) was found to be totally ineffective in restoring excitability of 4 axons examined. In all the cases, both the Na and K conductances were restored a little immediately after the internal perfusion solution was switched from the SIS to the "porcine brain-ligase." However, after that, the Na conductance was improved no more and the K conductance was gradually decreased. The leak conductance, resting potential and the reversal potential were not restored at all.

The "porcine brain-260K" (Table) was weakly effective in the restoration of 5 axons examined. In all the axons, it was observed that the excitability did not recover until the internal perfusion flow spontaneously stopped. While the axon was internally perfused with the "porcine brain-260K" at a usual flow rate of 10 to 20 µl/min after the 10 to 20 mM colchicine treatment, the Na and K currents continued to decrease. As a result, it usually took 4 to 15 min for restoration of the excitability and the degree of restoration was not large.

The largest recovery of excitability in the restoration experiments using porcine brain microtubule proteins was obtained for the axon internally perfused with "porcine brain-260K-ligase" (Table). Five axons were examined with this restoration system. One of the examples is illustrated in Fig. 12. The restoration took place immediately after switching the internal solution from the second SIS to the "porcine brain-260K-ligase." After a long time lag phase, Na conductance recovered more than K conductance. However, the leak conductance, resting potential and the reversal potential were not restored. This was a general feature commonly observed for restoration with porcine brain microtubule proteins. In contrast, in the restoration with squid brain or squid axoplasmic microtubule proteins, there was a general tendency for the leak conductance, resting potential and the reversal potential to improve, to greater or lesser extent, along with the Na and K currents.

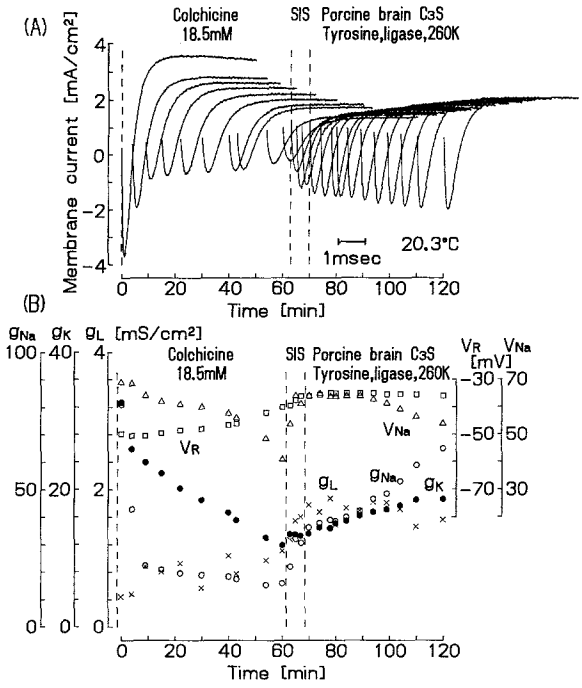

Fig. 12. Effect of the "porcine brain-260K-cAMP" on the restoration at 20.3 °C. The "porcine brain-260K-cAMP" was composed of 1 mg/ml porcine brain C_3S, 0.5 mM GTP, 3 mM $MgCl_2$, 3 U/ml tubulin-tyrosine ligase, 60 µM tyrosine, 1 mM ATP, 0.1 mg/ml 260K protein and the SIS (380 mM K, 355 mM F and 25 mM HEPES; pH 7.3). The squid giant axon immersed in ASW was intracellularly perfused with the SIS (until at 0 min), with the SIS containing 18.5 mM colchicine (from 0 through 61.5 min), with the SIS again (from 61.5 through 68.5 min), and with the "porcine brain-260K-cAMP," successively. (*A*) Changes of the membrane currents as a function of the perfusion time. The axon was voltage-clamped at membrane potential of 20 mV after preconditioned with a pulse of −100 mV for 30 msec. (*B*) Changes of the peak Na conductance g_{Na} (○), steady K conductance g_K (●), leak conductance g_L (×), resting potential V_R (□) and reversal potential V_{Na} (△), as a function of the perfusion time, respectively

Discussion

We have experimentally studied the effects of the reagents suppressing or supporting microtubule as-

sembly upon the various parameters in the Hodgkin-Huxley scheme (Hodgkin & Huxley, 1952). Among the reagents suppressing the assembly, we have examined colchicine, vinblastine, podophyllotoxin, sulfhydryl reagents such as DTNB and NEM, and chaotropic anions such as I^- and Br^- ions.

Colchicine specifically binds to a free tubulin dimer, resulting in suppressing elongation of microtubules (Margolis & Wilson, 1977). The binding depends on temperature; the higher the temperature is, the higher the binding probability becomes (Sherline, Leung & Kipnis, 1976). This may be one of the reasons why colchicine affected the Na current only at rather high concentrations (1 to 10 mM); that is, our experiments using colchicine were performed at temperatures below 20 °C, which was low compared with the temperature at which colchicine is usually used for biochemical experiments *in vitro* (around 37 °C). Temperature-dependent effectiveness of colchicine could explain the fact that colchicine suppresses Na current more strongly at higher temperatures than at lower temperatures (Fig. 4), and also that 0.1 to 1 mM podophyllotoxin strongly affected the Na current at lower temperatures (3 to 5 °C). It has been shown that podophyllotoxin specifically binds to the same site of a tubulin dimer as colchicine does, in a temperature-independent manner (Wilson, 1970; Wilson et al., 1974). Vinblastine also binds to a tubulin dimer at two sites, both of which are different from the site to which colchicine or podophyllotoxin binds, resulting in suppression of microtubule formation (Wilson, 1970; Wilson et al., 1974).

Modification of four cysteine residues of a tubulin dimer completely suppresses microtubule assembly (Kuriyama & Sakai, 1974). It was found that the effect of sulfhydryl reagents on the Na current was very similar to that of anti-mitotic drugs. This may be explained if the cysteine residues of axoplasmic tubulin dimers are more susceptible to chemical modification than those of other axoplasmic and membrane proteins. The overall effect of internal perfusion of a solution containing chaotropic anions upon the membrane currents was very similar to those of anti-mitotic drugs and sulfhydryl reagents. Chaotropic anions are considered to depolymerize microtubules into tubulins as a result of the weakening of hydrophobic interactions among tubulins (Sakai, 1980). Anti-chaotropic anions such as fluoride and glutamate have the opposite effect.

As a conclusion, all the experimental results on the effect of reagents specifically or nonspecifically suppressing microtubule assembly upon the Na, K and leak currents suggest that axoplasmic microtubules are mainly necessary for generation of the Na current. Particularly, the microtubules may be related to production of both maximum sodium conductance and sodium activation, but not related to sodium inactivation. However, it is still possible that axoplasmic microtubules and probably undercoat associated with the axolemma might produce, at least, some of their effects via slow inactivation on generation of Na current, in spite of the facts that voltage dependence of steady-state slow inactivation of the Na conductance were found similar for control and colchicine-treated axons. The above conclusion obtained from the pharmacological study is consistent with the finding by others (Eaton & Brodwick, 1975; Gillespie & Meves, 1980) that intracellular Ca^{2+} ions reduce the maximum Na conductance but do not affect the steady-state sodium inactivation and the time constant of the inactivation. It is well known that calmodulin (Cheung, 1970; Kakiuchi & Yamazaki, 1970) binds to microtubule proteins in a Ca^{2+} ion concentration-dependent manner, inducing microtubule disassembly (Marcum et al., 1978). The above conclusion is also consistent with the finding that millimolar Mn^{2+} or Mg^{2+} ions inside the axon, although they are both divalent ions, do not reduce electrical excitability (Eaton & Brodwick, 1975; Terakawa, Nagano & Watanabe, 1978; Matsumoto & Sakai, 1979*a*), since 0.1 to 0.5 mM Mg^{2+} ions are necessary for microtubule assembly (Olmsted & Borisy, 1975) and 1 mM Mn^{2+} ions do not harm the polymerization (Wallin, Larsson & Edstrom, 1977).

The conclusion obtained from the pharmacological study that axoplasmic microtubules are necessary for generation of Na current was further supported by just the opposite effect of the reagents supporting microtubule assembly (DMSO, Taxol, D_2O and temperature) to that of those suppressing the assembly on the Na conductance and Na activation. Tubulin dimers can be polymerized into microtubules in the presence of DMSO and/or Taxol, even in the absence of microtubule-associated-proteins (MAPs; Himes et al., 1976). Slow changes of the Na current in an axon bathed in a mixture of D_2O- and H_2O-ASW could be directly related to the axoplasmic microtubule assembly and disassembly. It has been found that the volume of spindle became maximum in the solution containing 45% D_2O (Inoué & Sato, 1967). This suggests that microtubules are best assembled in the 45% D_2O solution. Further, it is known that a 100% D_2O solution suppresses polymerization (Sato, Takahashi & Sata, 1980). As was seen in

Fig. 8, the peak Na current was most substantially restored in an axon bathed in 45% D_2O-ASW but peak Na current was reduced in 100% D_2O-ASW. The latter was also observed for the membrane currents of *Myxicola* giant axons by Schauf and Bullock (1980) and in squid giant axons by Meves (1974). It was also found that the effect of temperature on Na current (Fig. 9) could be explained in terms of microtubule assembly and disassembly; the peak Na conductance and the gradient of the m^3 curve were both maximum at a temperature around 25 °C, which corresponded to the optimal temperature for assembling axoplasmic microtubules in squid giant axons (Sakai & Matsumoto, 1978).

From the arguments described above, we suggest that axonal microtubules are necessary for generation of the Na current in squid giant axons. Further evidence for this suggestion was obtained from the restoration experiments. It was found that internal application of microtubule proteins, 260K protein and cAMP greatly restored the Na current if the following conditions were satisfied: (1) The microenvironment inside the axon is put under the conditions favorable for microtubule assembly; that is, anti-chaotropic anions such as fluoride and glutamate should be used in the internal perfusion medium (Sakai & Matsumoto, 1978), the temperature should be kept around 25 °C (Sakai & Matsumoto, 1978) for restoration and GTP and Mg^{2+} should be present in the perfusion medium (Weisenberg, Borisy & Taylor, 1968). In particular, it was experimentally confirmed that the internal medium devoid of GTP was ineffective for the restoration. These facts seem to exclude the possibility that tubulin incorporated into the membrane (Berlin, 1982) is related to generation of the Na current, and suggest that axoplasmic microtubules are important. (2) The 260K protein was found to be indispensable in producing stable restoration. Further, it was found that the 260K protein is capable of bundling microtubules as a cross-linker (Murofushi et al., 1983). These results suggest that axoplasmic microtubules associated with 260K proteins cross-link the axolemma. This kind of interaction may be necessary for generation of the Na current. (3) Tubulin with its C-terminus tyrosinated was found to be indispensable for the restoration. This was concluded from the results that the "porcine brain-260K" (devoid of tubulin-tyrosine ligase, tyrosine and the reagents activating the ligase) could not restore the membrane excitability.

As a conclusion of the restoration study, when the axon is partially reduced in its electrical excitability, internal application of microtubule proteins containing fully tyrosinated tubulin, 260K protein and cAMP into the axon could restore the Na conductance, resting potential and Na reversal potentials almost completely, and improve the K and leak conductances. This conclusion is consistent with the findings of the pharmacological studies mentioned above that internal perfusion with reagents suppressing microtubule assembly reduces the maximum Na conductance and shifts the curve of voltage dependence of the Na activation in a positive direction along the voltage axis, and that internal perfusion with reagents favorable for microtubule assembly causes just the opposite effect upon the maximum Na conductance and the Na activation. The importance of cAMP to restoration has been stressed repeatedly (Matsumoto & Sakai, 1979*b*; Matsumoto et al., 1982*a*) and was reconfirmed in the present restoration experiment. In our previous report (Matsumoto et al., 1982*b*), it was experimentally verified that phosphorylation might be important in maintaining the electrical excitability, and was conjectured that the phosphorylation might be cAMP-dependent and, therefore, important to maintain the excitability. We could not proceed further in elucidating the biochemical function of cAMP. This is left for the future work.

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