J. Membrane Biol. 120, 211–222 (1991)
002226319100048B

The Journal of
Membrane Biology

Anticalmodulin Drugs Block the Sodium Gating Current of Squid Giant Axons

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Summary. The effects of calmodulin (CaM) antagonists (W-7, W-5, trifluoperazine, chlorpromazine, quinacrine, diazepam, propericyazine and carmidazolium) on the sodium and potassium channels were studied on the intracellularly perfused and voltage-clamped giant axon of the squid. It was found that the drugs are more potent blockers of the sodium current than of the potassium current. The drugs also reduce the sodium gating current. The blockage of the sodium and gating current can be explained by assuming that the drugs interact with the sodium gating subunit in one of its closed states. The site of action is probably the intracellular surface of the axolemma where presumably a Ca^{2+}-calmodulin complex can be formed.

Key Words anticalmodulin drugs · trifluoperazine · W-7 · W-5 · sodium currents · sodium gating currents · squid giant axons

Introduction

The structural and functional organization of the sodium channels in myelinated or nomyelinated axons is currently of great interest. It seems likely that the α-polypeptide of the rat brain sodium channel is linked by ankyrin to the underlying cytoskeleton (Srinivasan et al., 1988). This suggests that the subaxolemmal cytoskeleton (Ellisman & Porter, 1980) may help to maintain and control the organization of the sodium channel in myelinated axons. In nonmyelinated axons of squid, the subaxolemmal cytoskeleton is highly differentiated (Kobayashi et al., 1986; Tsukita et al., 1986; Matsumoto, Tsukita & Arai, 1988); it is specialized into two domains (microtubule- and microfilament-associated domains) according to the underlying cytoskeleton. Electron microscopic observations on squid giant axons revealed that spectrin-like proteins linked between proteins protruded from the membrane and cytoplasmic actin filaments (Tsukita et al., 1986). This prompted us to study the effects of calmodulin inhibitors on the sodium and potassium currents of the squid giant axon. Antipsychotic or neuroplegic drugs like trifluoperazine, chlorpromazine or flurazepam are calmodulin inhibitors and their effect on the ionic currents of the nerve has been studied by previous authors. Seeman (1972) showed that chlorpromazine in 10-μM concentration blocks the action potential of a frog nerve. Flurazepam was tested on squid giant axons (Swenson, 1982) and on frog nerve (Schwarz & Spielmann, 1983). Externally applied flurazepam reduces both sodium and potassium current of squid giant axons and induces fast inactivation of potassium current, acting probably on the inner channel mouth. We have tested two structurally dissimilar groups of calmodulin inhibitors: (*i*) trifluoperazine and related antipsychotic drugs, and (*ii*) the sulfonamide derivatives W-5 and W-7. Some of the results described in the present paper were reported at the Annual Meeting of the Biophysical Society of Japan (Matsumoto & Urayama, 1985; Urayama & Matsumoto, 1986).

Materials and Methods

Materials

Giant axons of squid (*Doryteuthis bleekeri* or *Loligo budo*) were used. The squid were collected in Sagami Bay (Kanagawa, Japan), transported to the Electrotechnical Laboratory at Tsukuba City (Ibaraki, Japan), and maintained in a small, circular, closed-system aquarium until the experiments were performed (Matsumoto, 1976). The filtering system of the aquarium has been improved since 1984 (Matsumoto, 1985), by reducing the ammonium ion concentration in the aquarium seawater (NSW) to a point at or below 0.01 ppm. This could be realized by maintaining aerobic bacteria, which specifically eat ammonium, in the filter layer: charcoal with rather coarse pores was found to be the most suitable filtering material tested for the bacteria to settle on. Forty kg of charcoal was used for 1 ton of seawater. The filter was composed of three layers, with each layer being relatively thin (0.2–0.25 m) with a large surface area (about 2 m^2). Air was infused into the seawater ahead of each filter layer. To promote the growth of the bacteria, we continuously pumped ammonium ions (1 ppm) into the seawater for one week before the squid were introduced to the aquarium.

Table 1. Compositions and osmolarity of the internal and external solutions[a]

A. Internal solution

Name	K	TMA	F	Glutamate	Phosphate[d]	Tris[d]	Osmolarity
Standard internal solution (SIS)	400	—	—	380	20	—	~980[b]
400 KF	400	—	380	—	20	—	980[c]
200 TMA	—	200	50	150	—	10	970[c]

[a] Concentrations are in millimolar units, and osmolarity is in mosmol/kg.
[b] Osmolarity was adjusted by glycerol.
[c] Osmolarity was adjusted by sucrose.
[d] pH was adjusted to 7.2.

B. External solution

Name	Na	K	Ca	Cl	Trizma ~7.5	Osmolarity
H0 NaSW[b]	—	—	50	~675	~575[c]	1,100[e]
H1/10 NaSW[b]	55	—	50	~675	~520[c]	1,100[e]
1/10 NaSW	55	—	50	~595	~440[c]	978[e]
NaSW	420	10	40	520	10[d]	~980[f]
40Ca-NaSW	420	—	40	510	10[d]	~980[f]

[a] Concentrations are in millimolar units, and osmolarity is in mosmol/kg.
[b] Both solutions of H0 NaSW and H1/10 NaSW are hyperosmotic.
[c] pH was adjusted by Trizma −7.5 (Sigma Chemical) except NaSW.
[d] pH was adjusted to 8.2 for Tris.
[e] Osmolarity was adjusted by Tris (hydroxymethyl) aminomethane and Tris hydrochloride except NaSW.
[f] Osmolarity was adjusted by glycerol.

The squid axon diameters were between 300 and 500 μm. The majority of the adherent tissues surrounding the axon were removed under a dissecting microscope.

Experimental Procedures

The voltage-clamped and intracellularly perfused giant axon was used for the experiments. The methods of perfusion and voltage clamping were the same as those described in our preceding papers (Matsumoto, Ichikawa & Tasaki, 1984*a*; Matsumoto et al., 1984*b*). For the measurement of sodium and potassium currents, the conventional voltage-clamp method was adopted: the axon was usually held at −60 mV, given a prepulse to −100 mV for 30 msec and then depolarized or hyperpolarized to a test step for the membrane current measurement. The voltage-clamp system was similar in principle to the one developed by Bezanilla and Armstrong (1977). A compensated feedback system was adopted to reduce the error caused by the series resistance with the membrane (Katz & Schwartz, 1974). For the measurement of the gating current, on the other hand, the error caused by the series resistance was further reduced by decreasing the ionic concentration of seawater (*see* the Table). The extent of the compensation was adjusted for critical damping. As a result, the linear capacitive transient returned to the baseline within 18 μsec and did not exhibit a slow component, overshooting or ringing when a test potential of 60 mV in the negative direction was applied to the axon intra-axonally perfused with 200 tetramethylammonium (TMA) and bathed in the 0 Na seawater (*see* the Table). This confirmed the result obtained by Stimers, Bezanilla and Taylor (1987) that the Na gating current had no rising phase. The linear capacitive transient remaining after the above adjustment was reduced in size as far as possible by means of a transient subtracter (Bezanilla & Armstrong, 1977; Matsumoto et al., 1984*b*).

For the gating current measurement, we adopted a new pulse program of a family of eight positive test pulses with amplitudes progressively from $P/8$ to P in steps of $P/8$. During each 1,344-msec cycle, we applied the eight test pulses from a preholding potential (V_{PH}) of −70 mV interspersed with eight references pulses from a subtracting holding potential (V_{SH}) of −170 mV. For each 4-msec test and reference pulse we allowed 50 msec before and 30 msec after the pulse at the appropriate holding potential.

This pulse program provided the following advantages. (*i*) The voltage-dependent effects of drugs on gating currents was more precisely determined with this procedure than when we adopted the conventional pulse procedure, such as the $P/4$ procedure (Bezanilla & Armstrong, 1977; Matsumoto et al., 1984*a*) where the measurement was carried out using different cycles for each potential. In the present pulse procedure the drug effect was equally reflected on the measurements for different membrane potentials because all potentials are measured each cycle. (*ii*) The present pulse procedure can save measurement time since it occurred four times faster than that of the $P/4$ procedure. (*iii*) The small amplitude of the reference pulse (not exceeding 15 mV) can reduce errors that might be produced from the nonlinear effects which would occur if we were to use reference pulses with large

amplitudes (>30 mV). We experimentarily confirmed that the current records, as obtained using the present pulse procedure, were coincident with the those obtained using the *P*/4 procedure when the test pulse height, *P*, was below 105 mV. However, these records deviated from each other when *P* was above 105 mV. These deviations originated from the presence and absence of the nonlinearity. (*iv*) In the present procedure the amplitude of the reference pulse was 15 mV, irrespective of the test pulse height. Thus the reference pulse could escape from quantum noise accompanied by the digitization process which typically occurs in the current records for the test pulse amplitudes of 15–40 mV when we adopt the *P*/4 procedure. (*v*) The eight current records of reference pulses were added to eliminate the linear capacitive components of the records for the test pulses. This helped to improve the signal-to-noise ratio of the gating current records. To further improve the signal-to-noise ratio in the measured currents, we averaged either 32 or 64 cycles, and used a 2-pole Bessel filter (50 or 100 kHz at 3 dB).

Experiments were performed at temperatures between 9.5 and 18°C. The temperature of the external solution surrounding the axon was kept constant within ±0.05°C (Matsumoto et al., 1984*b*).

Solutions, Calmodulin Antagonists and Calmodulin

The compositions of both the internal and external solutions used in the present experiments are listed in the Table. The intracellular perfusion proceeded as follows: First, the giant axon was immersed in NaSW and intracellularly perfused with 400 KF. This was used for the ionic current measurement. Then, for the gating current measurement, the external and internal solutions were further switched to H1/10 NaSW or H0 NaSW, and 200 TMA, respectively.

The CaM antagonists used in these experiments were N-(6-aminohexyl)-1-naphthalensulfonamide hydrochloride (W-5), N-(6-aminohexyl)-5-chloro-1-naphthalensulfonamide hydrochloride (W-7), trifluoperazine, chlorpromazine, quinacrine, diazepam, propericyazine and carmidazolium (R24571). All the drugs were commercially available and used without any further purification: TMA was purchased from Sigma Chemical; W-5 and W-7 were from Seikagaku Kogyo; trifluoperazine, chlorpromazine, quinacrine, diazepam and propericyazine were obtained from Wako Pure Chemical Industries (Osaka, Japan); carmidazolium was from Janssen Life Sciences Products (Beerse, Belgium). CaM was purchased from Amano Chemical and used without any further purification.

In order to study the intracellular effects of the CaM antagonists on the electrical excitability, the squid giant axon was intracellularly perfused with an internal solution containing one of the drugs. Generally, intracellular perfusion was carried out using a silicon rubber tube. However, in the case of intracellular perfusion with a solution containing trifluoperazine, a Teflon tube was used to apply the internal perfusion fluid in order to avoid a reaction between the silicon rubber tube and the trifluoperazine.

Results

Effects of Trifluoperazine on Ionic Currents and the Sodium Gating Current

Trifluoperazine reduced the sodium current but had less effect on the potassium current. It had no appreciable effect on the leak current (*data not shown*). A typical experiment is illustrated in Fig. 1, where the squid giant axon immersed in ASW was intracellularly perfused with standard internal solution (SIS) (records *#1* and *6*) and SIS containing trifluoperazine (records *#2–5*) in succession. Trifluoperazine reduced the peak sodium current in a concentration-dependent manner (Fig. 2*A*) although 30–40 min were needed for the effect to reach steady state (Fig. 1). It was found that trifluoperazine was less effective in reducing the peak potassium current (Figs. 1 and 2*A*) but did have a significant effect on the potassium inactivation (insets of Fig. 1). Although we did not investigate the latter effect fur-

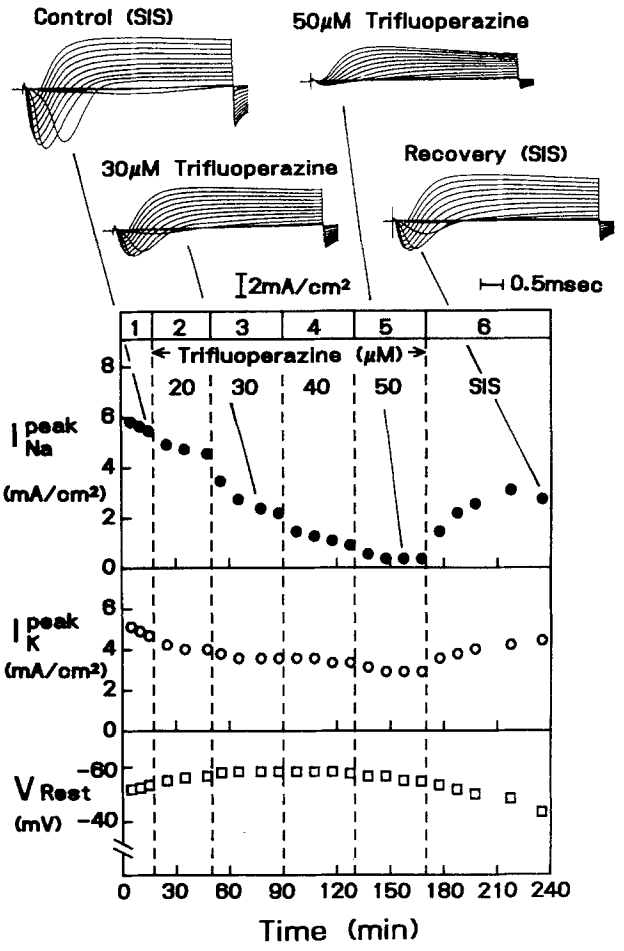

Fig. 1. Effects of intra-axonally applied trifluoperazine on sodium and potassium currents. The squid giant axon immersed in NaSW was intracellularly perfused with SIS containing 0 (records *#1* and *6*), 20 μM (record *#2*), 30 μM (record *#3*), 40 μM (record *#4*) and 50 μM (record *#5*) trifluoperazine. Peak sodium and potassium currents ($I_{Na^{peak}}$ and $I_{K^{peak}}$) for membrane potentials of −10 and 60 mV, respectively, and resting potentials (V_{Rest}) are shown as a function of perfusion time. *Inset:* The ionic currents obtained when the axon was voltage clamped to −60, −50, −40, −30, −20, −10, 0, 10, 20, 30, 40, 50 and 60 mV at the holding potential of −60 mV. Temperature was 16°C

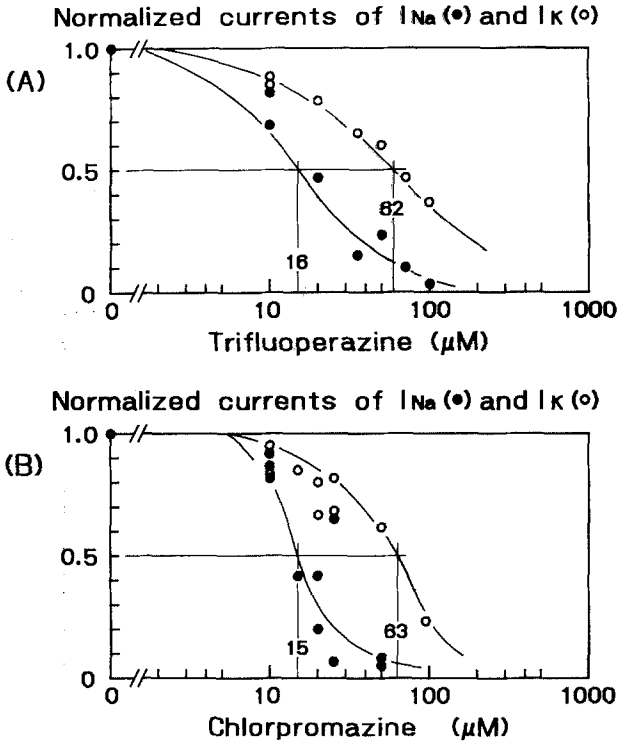

Fig. 2. Dose dependence of peak sodium (●) or potassium (○) currents when trifluoperazine (*A*) or chlorpromazine (*B*) was internally applied. Peak sodium or potassium currents at membrane potentials of −10 and 60 mV, respectively, were normalized by those obtained without the drug

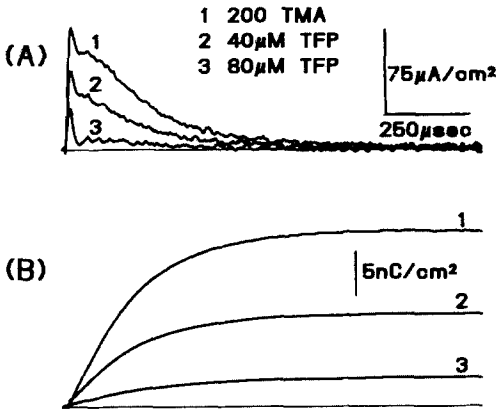

Fig. 3. Effects of internal trifluoperazine (TFP) on the gating current. (*A*) Gating current records obtained when the axon bathed in H0 NaSW was internally perfused with 200 TMA (record *#1*), and 200 TMA containing 40 μM (record *#2*; 35 min) and 80 μM TFP (record *#3*; 26 min), in succession, where the time in parentheses represents the elapsed perfusion time after switching the internal solution. (*B*) Running integrals of the corresponding gating current records. Temperature, 10.2°C. Test pulse amplitude, 105 mV. V_H, −60 mV. V_{PH}, −70 mV. V_{SH}, −170 mV. Averaging number, 32. Cut off frequency of the Bessel filter, 50 kHz

ther, concentration-dependent acceleration of the potassium inactivation is the characteristic most commonly observed in the effects of flurazepam on the potassium current (Swenson, 1982; *see also* Fig. 5). The effects of trifluoperazine on the sodium and potassium currents were partially reversible (*see* record *#6* in Fig. 1).

Effects of trifluoperazine on the sodium gating were studied in detail by measuring the sodium gating current. Trifluoperazine reduced the sodium gating current in a concentration-dependent manner. Typical records of the effect from the axon bathed in H0 NaSW are illustrated in Fig. 3 where the test pulse of 105 mV was applied to the axon internally perfused with 200 TMA (record *#1*) and with 200 TMA containing 40 μM (record *#2*) and 80 μM (record *#3*) trifluoperazine (TFP). Running integrals Qg of the gating currents (Ig) thus obtained were calculated by the relation

$$Qg(t) \equiv \int_o^t Ig(\tau)\, d\tau$$

(Keynes, Greeff & Van Helden, 1982; Matsumoto & Ichikawa, 1985), where t represents the time measured from the start of the test potential step application. Plots of the running integrals corresponding to gating current records *#1–3* are shown in Fig. 3*B*. It is seen in Fig. 3 that (*i*) TFP reduces the charge carried by the gating current in a concentration-dependent manner, and that (*ii*) the time course of the falling phase in the gating current changes with the TFP treatment, but (*iii*) the time course of the rising phase does not change with the treatment and exhibit an instantaneous rise time (Stimers et al., 1987). Further, it is noted that the falling phase of the gating current for the control axon, that is the axon intracellularly perfused with 200 TMA, does not show exponential decay (record *#1* in Fig. 3*A*; *see also* Matsumoto et al., 1984*a*; Matsumoto & Ichikawa, 1985). This is in sharp contrast with the falling phase of the gating current obtained by using tetrodotoxin (TTX) in the external medium for the control axon (for example, *see* Stimers, Bezanilla & Taylor, 1985; Stimers et al., 1987). The gating current after the TTX treatment decays exponentially with two or three time constants of the decay. The difference in the falling phase will be discussed in greater detail in terms of the interaction of TTX with the sodium channels in a separate paper (Ichikawa & Matsumoto).[1]

[1] Ichikawa, M., Matsumoto, G. Tetrodotoxin affects sodium gating currents in squid axons. (*unpublished manuscript*)

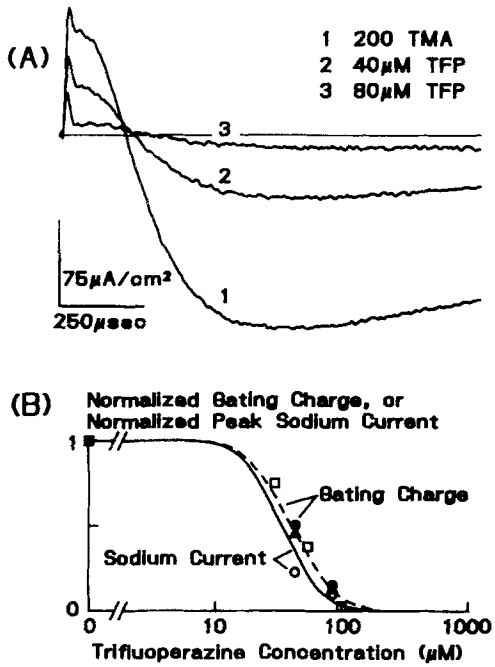

Fig. 4. Effects of internal trifluoperazine (TFP) on both the gating and sodium currents. (*A*) Current records obtained when the axon bathed in H1/10 NaSW was internally perfused with 200 TMA (record *#1*), and 200 TMA containing 40 μM (record *#2*; 20 min) and 80 μM (record *#3*; 20 min), in succession, where the time in parentheses represents the elapsed perfusion time after switching the internal solution. Temperature, 10.2°C. Test pulse amplitude, 105 mV. V_H, −60 mV. V_{PH}, −70 mV. V_{SH}, −170 mV. Averaging number, 32. Cut off frequency of the Bessel filter, 50 kHz. (*B*) TFP concentration dependence on normalized peak sodium current (solid line, open symbols) and normalized gating charge (dotted line, filled symbols). The relation between the gating charge *vs.* TFP obtained was as shown in Fig. 3. The TFP dependence on the peak sodium current was obtained as shown above and had been outlined in detail in Fig. 2*A*. The different symbols are different axons

Reduction of the gating charge by the TFP treatment was found to correlate with the blocking of the sodium current: a typical experiment showing this is illustrated in Fig. 4*A* where the same axon as that used in Fig. 3 was bathed in H1/10 NaSW and internally perfused with 200 TMA (record *#1*), 200 TMA containing 40 μM (records *#2*) and 80 μM (record *#3*) TFP, in succession. A test pulse of 105 mV was superimposed on the preholding potential of −70 mV, as shown in Fig. 3. It was found that the reduction of the gating charge by the TFP treatment correlated well with the blockage of the sodium current (Fig. 4*B*). These results indicate that TFP reduced the gating current in a concentration-dependent manner, resulting in blockage of the sodium current. In other words, the interaction site of TFP for the sodium channel was on the sodium gate but not in the sodium pore. These can be explained by assuming that TFP interacts with the sodium gate to inhibit the transition between the initial two gate states corresponding to closed channels, thus resulting in the inhibition of all following transitions (*see* Discussion).

Among other anti-calmodulin drugs similar to trifluoperazine, chlorpromazine, quinacrine, diazepam and propericyazine were tested. Effects of chlorpromazine on the sodium and potassium currents were found to be particularly similar to those of trifluoperazine; that is, (*i*) chlorpromazine was more effective in reducing the peak sodium current in a concentration-dependent manner than in reducing the peak potassium current (Fig. 2*B*). (*ii*) Chlorpromazine did alter the potassium inactivation in a concentration-dependent manner. (*iii*) All of these effects were partially reversible. The effects of either quinacrine, diazepam or propericyazine were qualitatively similar to those of trifluoperazine or chlorpromazine, except for their dose dependencies. The concentrations of the drug needed to block half of the control potassium current ($I_{K^{1/2}}$) were higher than those needed to block half of the control sodium current ($I_{Na^{1/2}}$): ($I_{Na^{1/2}}$ and $I_{K^{1/2}}$) were (105 μM, >50,000 μM), (150 μM, 320 μM) and (85 μM, 140 μM) for quinacrine, diazepam and propericyazine, respectively. These findings suggest that these anticalmodulin drugs were bound to the same site(s), thus inducing similar physiological effects.

W-7 AND W-5

W-7, a structurally dissimilar calmodulin inhibitor to trifluoperazine, was tested to examine its effect on the sodium and potassium channels. In this experiment, the effect was compared with that of W-5, a dechlorinated analog of W-7, since W-5 is less potent in the inhibition of CaM-induced activation of phosphodiesterase than W-7 (Asano & Hidaka, 1984). It was found that the effects of both W-7 and W-5 on sodium and potassium currents, when intra-axonally applied, resembled those of trifluoperazine. The effects of W-7 are shown in Fig. 5 where the voltage-dependent membrane currents are recorded for the axon intra-axonally perfused with SIS (record #1*A*) and SIS containing 20 μM (record *#2A*), 35 μM (record *#3A*), 50 μM (record *#4A*) and 75 μM (record *#5A*) of W-7, in succession. The records were all taken 5 min after the perfusion fluid was switched. The effects of W-7 reach a steady state more rapidly than trifluoperazine. It was found that much more time (20–30 min) was needed for recovery (record *#3B*) once the sodium and potassium currents were completely blocked by perfusing the axon intra-axo-

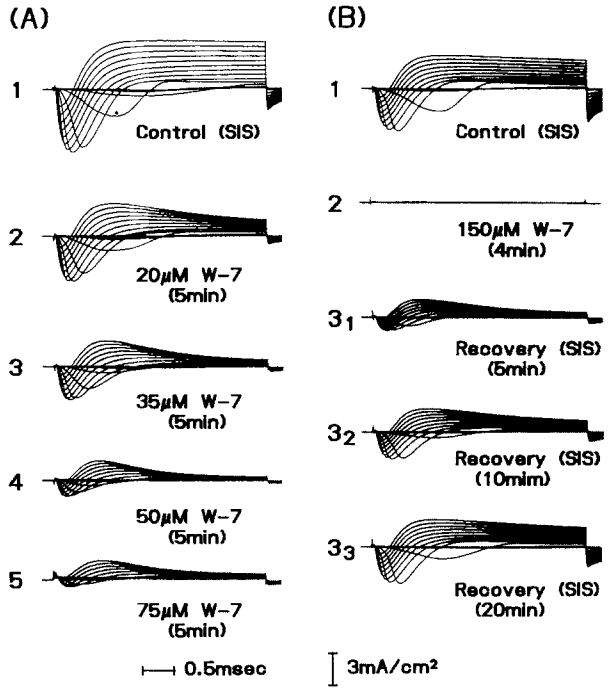

Fig. 5. (*A*) Dose-dependent blockage of sodium and potassium currents after the axon bathed in NaSW was intra-axonally perfused with SIS (record *#1*) and SIS containing 20 μM (record *#2*), 35 μM (record *#3*), 50 μM (record *#4*) and 75 μM (record *#5*) of W-7, in succession. The membrane currents were measured for the potentials of −60, −50, −40, −30, −20, −10, 0, 10, 20, 30, 40, 50, 60 and 70 mV when the axon was held at −60 mV. The time in the parenthesis for each record stands for the time measured after the perfusion fluid for the record is switched. (*B*) Recovery of sodium and potassium currents once after they were completely blocked by intra-axonal perfusion with SIS containing 150 μM W-7 (*see* record *#2*). Record *#3* represents the recovery. The time in parentheses represents the elapsed perfusion time after switching the internal solution. Temperature, 16°C

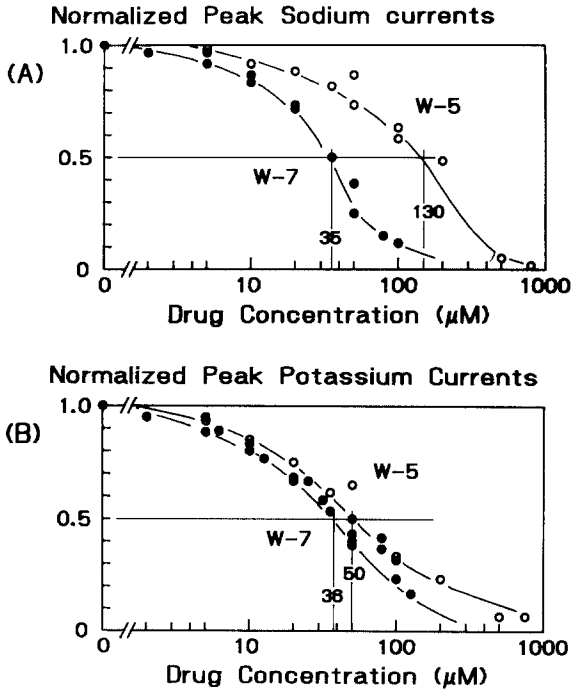

Fig. 6. Dose dependence on the blockage of the peak sodium (*A*) or potassium (*B*) current when the axon was intra-axonally perfused with SIS containing W-7 (●) or W-5 (○). Temperature, 16°C

nally with SIS containing 150 μM W-7 (record *#2B*). The concentration dependence of the blockage of the sodium current was found to differ significantly between the internal application of W-7 and that of W-5 (Fig. 6*A*): 35 μM of W-7 reduced the peak sodium current to half of its control value, while 130 μM of W-5 was needed for the same amount of reduction. Thus, W-7 was four times as potent in the inhibition of the sodium channel activity. The dose dependence of the blockage of the potassium current showed only a slight difference between W-7 and W-5 (Fig. 6*B*): the peak potassium current was reduced to half of its control value by 36 μM of W-7 or 50 μM of W-5. These findings indicate that the action of the CaM antagonists to the sodium channel might be more specific than those to the potassium channel. This hypothesis is also supported by the fact that the doses of W-7 or W-5 necessary for the inhibition of the sodium channel activity were closer to those needed for the inhibition of the CaM-dependent phosphodiesterase activity (Asano & Hidaka, 1984; *see also* Fig. 10) than those necessary for the inhibition of the potassium channel.

The effects of internal W-7 on the sodium gating current were also studied in detail by the same experimental procedures as the records in Figs. 3 and 4 for the effect of trifluoperazine. A typical experiment is illustrated in Fig. 7*A* where the axon bathed in H0 NaSW was internally perfused with 200 TMA (record *#1*), and with 200 TMA containing 12.5 μM (record *#2*), 25 μM (record *#3*), 50 μM (record *#4*) and 100 μM (record *#5*) of W-7, in succession. The test pulse of 105 mV was superimposed on the pre-holding potential of −70 mV. As seen in Fig. 7*A*, W-7 reduced the sodium gating current in a concentration-dependent manner. The running integrals of these gating currents were obtained as shown in Fig. 7*B*, and the W-7 concentration dependence of the charges carried by the gating currents were obtained and compared with the dependence of the peak sodium currents (Fig. 7*C*). The concentration dependence of the gating currents deviated from that of the sodium current; that is, 100 μM W-7 blocked the sodium current completely but inhibited the gating current partially (see the gating current records in

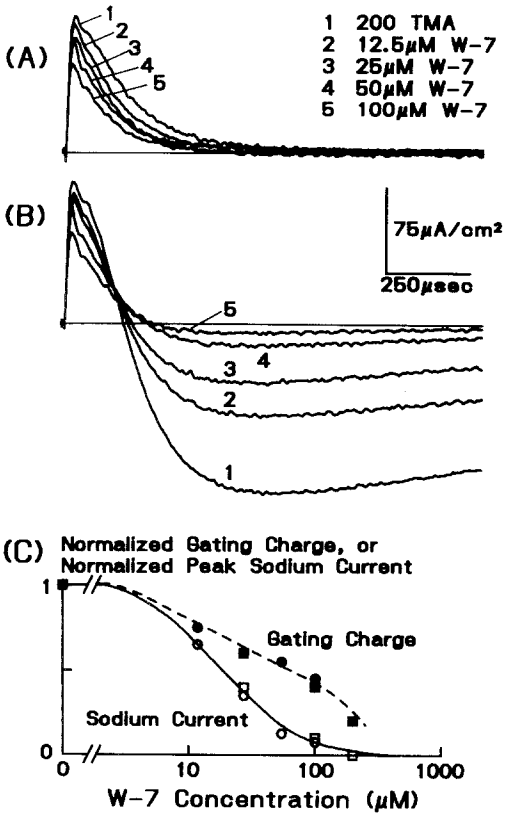

Fig. 7. Effects of internal W-7 on the gating current. (*A*) The concentration-dependent block of the gating current. The axon bathed in H0 NaSW was internally perfused with 200 TMA (record *#1*), and with 200 TMA containing 12.5 μM (record *#2*), 25 μM (record *#3*), 50 μM (record *#4*) and 100 μM (record *#5*) W-7, in succession. The measurement for each drug concentration was made 20 min after switching the solution. Temperature, 9.6°C. Test pulse amplitude, 105 mV. V_H, −60 mV. V_{PH}, −70 mV. V_{SH}, −170 mV. Averaging number, 32. Cut frequency of the Bessel filter, 50 kHz. (*B*) Current records obtained when the axon bathed in H1/10 NaSW was internally perfused with 200 TMA (record *#1*), and 200 TMA containing 12.5 μM (record *#2*), 25 μM (record *#3*), 50 μM (record *#4*) and 100 μM (record *#5*), in succession. (*C*) W-7 concentration dependence of normalized peak sodium current (solid line, open symbols) and normalized gating charge (dotted line, filled symbols)

Fig. 7*A*, and also the drug potency on the inhibition to the sodium ionic and gating currents in Fig. 7*C*). Effects of W-5 on the gating current were qualitatively similar to those of W-7, except its drug potency. The concentration of W-5 needed to reduce the gating charge $Q_{g1/2}$ to one-half of the control value (the gating charge obtained without W-5) was 300 μM, while that of W-7 was 70 μM. Therefore, W-7 is four times more potent in inhibiting the gating charge than W-5. The falling phase of the gating current decayed exponentially as the concentration of W-5 applied to the axon was increased. The same

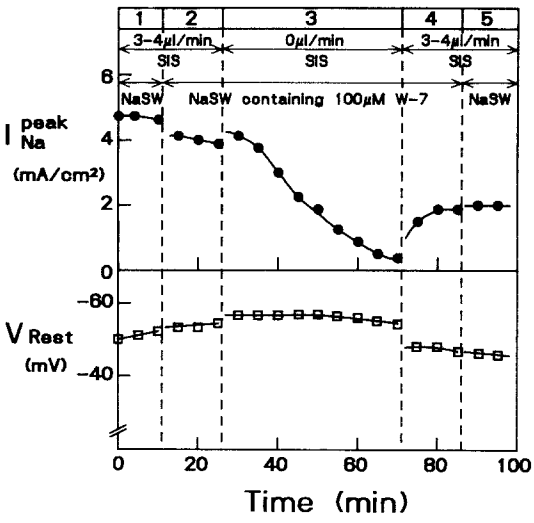

Fig. 8. Effects of both external application of W-7 and internal perfusion velocity on the peak sodium current and resting potential as a function of the perfusion time. The velocity of the internal perfusion fluid (SIS) was set at 3–4 μl/min (records *#1, 2, 4* and *5*) or 0 μl/min (the perfusion stopped; record *#3*). The axon was bathed in 40 Ca-NaSW (records *#1* and *5*) or 40 Ca-NaSW containing 100 μM of W-7 (records *#2–4*). NaSW in the figure is the abbreviated form of Ca-NaSW. Temperature, 16°C

was found for the W-7 application, as seen in Fig. 7*A*).

Effects of Externally Applied W-7 on the Sodium and Potassium Currents

So far we described effects of internally applied calmodulin inhibitors on the sodium and potassium channels. Here we tested the effects of externally applied W-7 when the giant axon was intracellularly perfused with SIS. An example of this experiment is illustrated in Fig. 8 where the axon was bathed in 40 Ca-NaSW or 40 Ca-NaSW containing 100 μM W-7 (which is a sufficient amount to block the sodium current almost completely if applied intra-axonally as shown in Fig. 6) while the axon was intra-axonally perfused with SIS at the usual velocity of 3–4 μl/min (records *#1, 2, 4* and *5*) or without any flow of the fluid (record *#3*). It was found that 100 μM of W-7 externally applied elicited only a 20% reduction in the peak sodium current when the internal perfusion fluid was at the usual flow velocity (record *#2* in Fig. 8), but that it reduced the sodium current, as if it were intra-axonally applied, when the perfusion fluid was stopped (record *#3*). The 45 min it took to reach the saturation point was considerably longer than the 5–10 min needed when it was internally applied. A partial recovery in the sodium current was observed when the perfusion

fluid was again applied at the usual flow velocity, even when the axon was still immersed in 40 Ca-NaSW containing 100 μM of W-7 (record *#4*). The degree of recovery was not improved when the external medium was switched to 40 Ca-NaSW without W-7 (record *#5*). These results indicate that the action sites of W-7 are inside the axon. This is similar to the conclusion by Swenson (1982) on the effect of flurazepam on the potassium current block of squid giant axon since internally applied flurazepam was found to be 1.5 times more potent than when applied externally. When W-7 penetrated through the membrane inside the axon and was eluted out by the internal perfusion fluid, its effect to reduce the sodium conductance was extremely weakened. In turn, if the penetrated W-7 was not eluted out and accumulated inside the axon, it was as effective in reducing the sodium current as if it were internally applied. However, it would take much more time for the effect of the externally applied W-7 to become saturated than when internally applied. This would explain the results of the experiments in which the saturation level of the 100 μM of W-7 was the same for both the internal and external applications and that the times needed to reach the saturation level were different between the external and internal applications. These findings suggest that the action site of W-7 is not located in the membrane, but on the cytoplasmic surface of the membrane. Supporting this view are experimental results showing that the recovery level was not improved by substituting the external medium of 40 Ca-NaSW for 40 Ca-NaSW containing 100 μM of W-7.

Effects of Simultaneous Application of W-7 with Calmodulin on the Sodium Current

The axon was intra-axonally perfused with SIS containing both 35 μM of W-7 and CaM, where the CaM concentration was changed from 0 through 150 μM. It was found that the blockage of the sodium current caused by the 35 μM of W-7 was removed by the exogenous CaM (Fig. 9). The extent of the removal depended on the CaM concentration; about 350 μM of CaM was estimated to be necessary to completely remove the effect caused by 35 μM of W-7. These results support a possibility that exogenous CaM could bind to intracellular Ca^{2+} ions to form the Ca^{2+}-CaM complex in the case of intracellular perfusion of SIS, since W-7 was known to specifically interact with this complex (Asano & Hidaka, 1984), inducing the reduction in potency of the W-7 blockage of the sodium current.

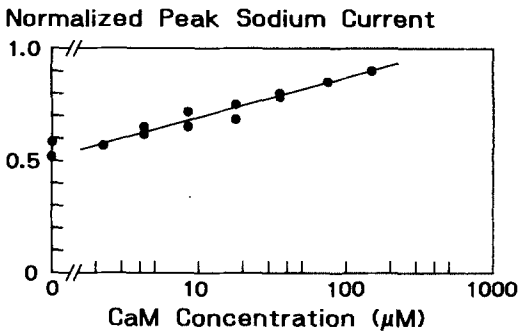

Fig. 9. Removal for the blockage of the sodium current caused by internal perfusion with SIS containing 35 μM of W-7, by exogenous calmodulin. The axon was voltage clamped at −20 mV and intra-axonally perfused with SIS containing 35 μM of W-7 and calmodulin where the calmodulin concentration was changed from 0 through 150 μM. Temperature, 16°C

Carmidazolium (R24571)

Carmidazolium is an anti-calmodulin drug which is structurally dissimilar from the phenothiazines (trifluoperazine, chlorpromazine), the diazepines and W-5/W-7. Two to 5 μM carmidazolium blocked both the sodium and potassium currents completely but simultaneously increased the leak current significantly. Appearance of the large leak current prevented us from being able to measure the precise effects of the drug.

Discussion

Effects of the CaM Antagonist on Sodium and Potassium Channels and Their Specificity

Each CaM antagonist tested in this study has affected both the sodium and potassium channels. In the sodium channel we found that these antagonists reduce the maximum sodium conductance in a concentration-dependent manner. In the potassium channel they reduce the potassium conductance and change the potassium inactivation, both in a concentration-dependent manner. These results qualitatively confirm previous experiments done by Swenson (1982) and also by Schwarz and Spielman (1983) on the effects of flurazepam upon squid giant axons and frog nerve, respectively, since flurazepam is a benzodiazepine similar to diazepam.

We found that during a 6-msec pulse the effects on the sodium channel are more dramatic than those on the potassium channel. This is in contrast with the effect of flurazepam on steady-state currents in

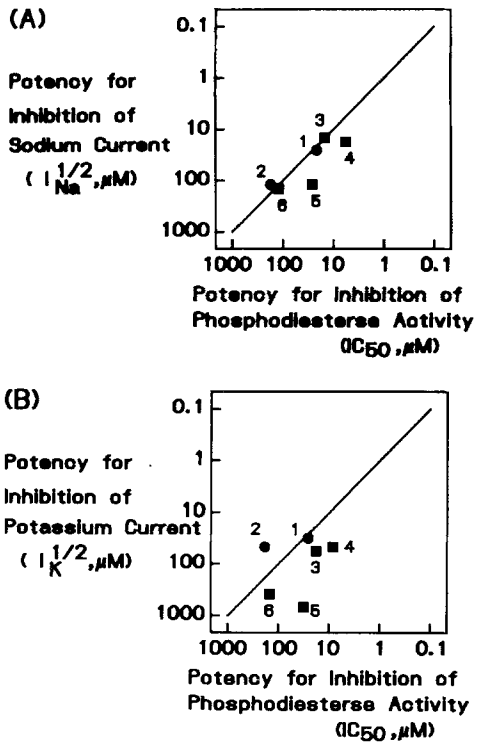

Fig. 10. Correlations between potency of calmodulin antagonists for inhibition of sodium current and that for phosphodiesterase activity (*A*), and between potency of calmodulin antagonists for inhibition of the potassium current and that for phosphodiesterase activity (*B*). The points represent W-7 (*1*), W-5 (*2*), chlorpromazine (*3*), trifluoperazine (*4*), quinacrine (*5*) and diazepam (*6*), respectively

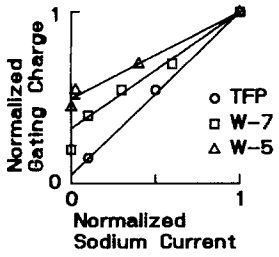

Fig. 11. Correlation between the inhibition, caused by the calmodulin antagonists (trifluoperazine, W-7 and W-5), of the sodium gating charge and of the sodium current. This correlation diagram was prepared based on the results with 40/80 μM TFP from Fig. 4*B*, 12.5–100 μM W-7 from Fig. 7*C* and the similar experiment with W-5 (the original data are not shown)

that flurazepam was three times a more potent blocker to potassium current than to sodium current in squid giant axons (Swenson, 1982). The drug concentration needed to reduce the sodium or potassium current to one-half of the control level (the current elicited without application of the drug) is summarized in Fig. 10 by comparing it with the potency of CaM antagonists necessary for inhibition of phosphodiesterase activity (Asano & Hidaka, 1984). The potency of the CaM antagonists (W-7, W-5, trifluoperazine, chlorpromazine, quinacrine and diazepam) for inhibition of phosphodiesterase activity is similar to that for the inhibition of the sodium current (Fig. 10*A*), but dissimilar to that for the inhibition of the potassium current (Fig. 10*B*). In Fig. 10*A*, the points for the CaM antagonist potency are located on or close to the line, exhibiting excellent correlation. In Fig. 10*B*, on the other hand, the points are further scattered around the line. These results suggest the possibility that the effect of the CaM antagonists on the sodium channel are induced by a specific interaction between the antagonists and the Ca^{2+}-CaM complex, but that the effect on the potassium channel may be due to some side effects caused by the CaM antagonists (Asano & Hidaka, 1984).

The specific blockage of the sodium current was found to be originated from the specific interaction between the calmodulin inhibitor and the sodium gating subunit. This was concluded from the experiments that (*i*) either TFP, W-7 or W-5 blocked both the sodium ionic and gating currents and that (*ii*) there was a close relationship between the sodium current blockage and the sodium gating current inhibition, as shown in the correlation diagram (Fig. 11) prepared from the effects of each of these three antagonists: Figure 4*B* (the TFP effects), Fig. 7*C* (the W-7 effects) and the W-5 effects (the original data are not shown). In Fig. 11, it was found that the inhibition of the gating current was linearly correlated to the blockage of the sodium current. The TFP-treated axon exhibited the perfect correlation, while, in the W-7 or W-5 treated axon, complete blockage of the sodium current took place with an appreciable amount of the sodium gating current remaining. The difference in the potencies of the CaM antagonists on the effects of the sodium current *versus* the gating current could be explained on the multi-state model proposed by Bezanilla and his colleagues. Bezanilla, Taylor and Fernandez (1982) and Stimers et al. (1985, 1987) proposed a kinetic model of the sodium channel, in which the gating process had seven states (a single open state with six closed states) regulated by the membrane potentials. The sodium channel opened if the gate arrived at the final open state through a series of successive transitions to the other six closed states. Based upon this model, both the sodium and gating currents would be simultaneously and completely suppressed if a specific reagent could inhibit the transition between the first and second closed states. This may be the case for the effect of trifluoperazine on the sodium channel of squid giant axons (Fig. 12). Yet, if a reagent could

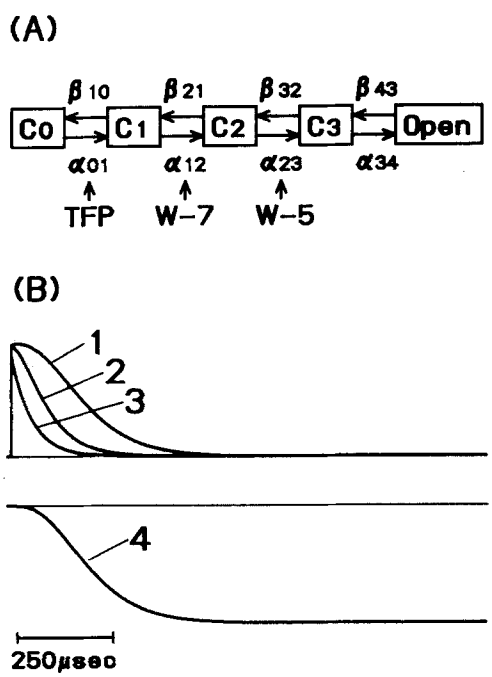

Fig. 12. (*A*) A schematic diagram representing five states of the sodium gate (four closed states, C_0, C_1, C_2 and C_3, and one open state, *Open*) and action sites of the drug (TFP, W-7, W-5) to block the transition between the neighboring states. It was assumed that TFP (trifluoperazine), W-7 and W-5 block the transition between C_0 and C_1, between C_1 and C_2, and between C_2 and C_3, respectively (*see text* for details). (*B*) Simulation results of the gating current calculated on the above model, *A*. The following are assumed: Initial values; $C_0 = 0.9$, $C_1 = 0.08$, $C_2 = 0.02$, $C_3 = Open = 0$. Transition velocities; $\alpha_{01} = \alpha_{23} \times 1.1$, $\alpha_{12} = \alpha_{23} \times 1.2$, $\alpha_{34} = \alpha_{23} = 17.1$ (msec)$^{-1}$, $\beta_{10} = \beta_{21} = \beta_{32} = \beta_{43} = 0$. Record *1;* total gate current (control). Record *2;* the gating current obtained as the transition is inhibited between C_2 and C_3, corresponding to the W-5 treated gating current (i.e., the gating current obtained when the sodium current was blocked by internally applied W-5). Record *3;* the gating current obtained as the transition is inhibited between C_1 and C_2, corresponding to the W-7 treated gating current. No gating current is observed when the sodium current is blocked in the TFP-treated axon since the transition is blocked between C_0 and C_1. Record *4* represents the running integral of record *1* (the open-state fraction) as a function of time

inhibit the second-to-third or any other transition higher than the first-to-second transition, the sodium current would be completely blocked but the gating charge could still be displaced upon depolarizing of the membrane. This is due to the fact that transition of the gate, for example to the second state, can occur upon depolarization even if the drug inhibits the transition to the third state and the channel cannot open. This may be the case of the effect of W-7 (Fig. 12*A*). A simulated gating current after the W-7 treatment is illustrated in record *3* of Fig. 12*B*. Based on this model, the effect of W-5 on the sodium gating current was also simulated when W-5 was assumed to inhibit the transition to the fourth closed state, as shown in record *2* of Fig. 12*B*. This assumption was made because the gating charge fractions remained finite with larger amounts remaining for the W-5 treatment than for the W-7 treatment after the sodium current was completely blocked (*see* Fig. 11).

Thus, the present experiments suggest that the CaM antagonists bind the sodium gate in the closed state, and that the affinity of an antagonist to the closed gate state differs from drug to drug; TFP can interact with the gate in the first closed state with very high affinity, followed by inhibition of the transition to the second closed state. On the other hand, W-7 and W-5 may interact with the gate in the second and third closed states, respectively, with higher affinity than with that in the first closed state.

How Do the CaM Antagonists Block the Sodium Gating?

From the above a possible explanation has been drawn that the CaM antagonists phenomenologically block the sodium gating. Then, a question arises how the antagonists block the gating. It should be acknowledged that the pharmacological effects of the CaM antagonist in the cell should be carefully analyzed (Tanaka, Ohmura & Hidaka, 1982; Wise et al., 1982; Dillingham et al., 1986). In fact, it is well known that calmodulin antagonists inhibit Ca-activated potassium conductance (Lackington & Orrego, 1981; Okada et al., 1987) and that they reduce the open probability and increase flickering noise (McCann & Welsh, 1987; Tokuno et al., 1988). However, it is still unclear how the Ca^{2+}-CaM complex regulates the activation process of the Ca^{2+}-induced channel (McCann & Welsh, 1987). The following experimental facts might support us to discuss the possibility that the blockage of the sodium current by the CaM antagonist might be caused by its specific interaction with the Ca^{2+}-CaM complex for the following four reasons: (*i*) Either of two structurally dissimilar CaM antagonists (trifluoperazine and W-7) had similar effects on the sodium channel. The same argument was utilized to get the result that calmodulin might be an important mediator of the hydrosmotic response to arginine vasopressin (Dillingham et al., 1986). (*ii*) The concentration of the drug needed to block 50% of the sodium current was close to that needed to inhibit CaM-dependent phosphodiesterase activity. In particular, both W-7 and W-5 were similar in their effects, but W-7 was more potent than W-5 as expected from their inhibition of CaM-induced activation of phosphodiesterase activity. (*iii*) The interaction site of the drug

was on the cytoplasmic side of the axolemma. (*iv*) Reduction of the effect by added CaM indicates that a Ca^{2+}-CaM complex could be formed in the subaxolemmal region inside the squid axon because this complex has been shown to interact with these drugs in vitro (Asano & Hidaka, 1984).

Although these four reasons cannot eliminate other possibilities about the site of the inhibition of the sodium gating (Moore & Dedman, 1981; Dillingham et al., 1986), these experiments, at least, do not eliminate a possibility that calmodulin may play an important role as a regulator of the sodium gating in the squid giant axon. Together with the electron microscope observation of the underlying structure of the squid axolemma (Tsukita et al., 1986; Matsumoto et al., 1988), which showed us that spectrin-like proteins linked between proteins protruded from the membrane and cytoplasmic actin filaments, these findings may allow us to theorize that spectrin (a CaM-binding protein) could possibly bind to the gating subunits of the sodium channel protein and regulate the channel gating function.

The authors would like to express their thanks to Dr. Shoichiro Tsukita (Tokyo Metropolitan Institute of Medical Science), Dr. Takao Arai (University of Tsukuba), Prof. Yoshio Watanabe (University of Tsukuba) and Dr. Junko Ohnishi (National Institute of Health), for their stimulating discussions. They also acknowledge Mr. Y. Kawakami (Stanley Co.) and Mr. H. Takemura (Nihon-Ricoh Medical Laboratory) for their help during the initial stage of the present experiments.

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Received 19 June 1990; revised 25 September 1990