J. Physiol. (1974), **242**, *pp.* 471–487
With 9 text-figures
Printed in Great Britain

MECHANISM OF NERVE MEMBRANE DEPOLARIZATION CAUSED BY GRAYANOTOXIN I

BY TOSHIO NARAHASHI AND ISSEI SEYAMA*

From the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, U.S.A.

(*Received 9 April 1974*)

SUMMARY

1. The mechanism of depolarization of squid axon membranes caused by grayanotoxin I has been studied by means of internal perfusion and voltage clamp techniques.

2. The depolarization induced by either internal or external application of grayanotoxin I was reversed by decreasing the external sodium concentration from 449 to 1 mM.

3. No depolarization was observed when both external and internal media were devoid of sodium ions, indicating that the depolarization by grayanotoxin I in normal media is due to a specific increase in resting sodium permeability.

4. The resting sodium permeability as measured by voltage clamp was increased to $1{\cdot}31 \times 10^{-6}$ cm/sec by internal application of 1×10^{-5} M grayanotoxin I, an increase by a factor of about 90.

5. The apparent dissociation constant of internally applied grayanotoxin I in increasing the resting sodium permeability was estimated to be $4{\cdot}12 \times 10^{-5}$ M, and the toxin interacts with the membrane receptor on a one-to-one stoichiometric basis.

6. Tetrodotoxin antagonized the action of grayanotoxin I in increasing the resting sodium permeability in a non-competitive manner.

INTRODUCTION

Several compounds have been found to cause a depolarization of nerve and muscle membranes through a specific increase in resting permeability to sodium ions. For example, veratridine has a potent depolarizing action on nerve membranes, and the effect is reversed by a drastic decrease in external sodium concentration or by an addition of tetrodotoxin to external medium (Ulbricht, 1969; Ohta, Narahashi & Keeler, 1973). Batrachotoxin

* Present address: Department of Physiology, School of Medicine, Hiroshima University, Hiroshima, Japan.

causes an irreversible depolarization in squid axon membranes without affecting the mechanism responsible for generation of the action potential (Narahashi, Albuquerque & Deguchi, 1971; Albuquerque, Seyama & Narahashi, 1973).

Grayanotoxins are contained in the leaves of the plants belonging to the family Ericaceae. The toxins contain several analogues, and their chemical structures have been completely identified (Kakisawa, Kurono, Nakanishi & Hirata, 1961; Kakisawa, Kozima, Yanai & Nakanishi, 1965;

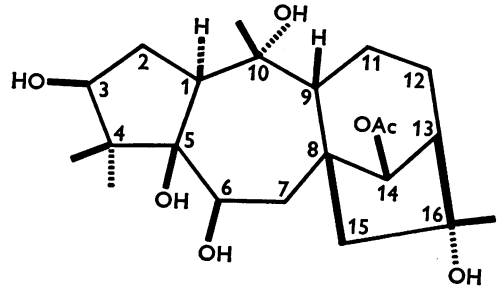

Fig. 1. Chemical structure of grayanotoxin I.

Kozima, Nakanishi, Yanai & Kakisawa, 1964; Kumazawa & Iriye, 1970; Matsumoto & Watanabe, 1968). Grayanotoxin I (Fig. 1) depolarizes the skeletal muscle membrane, and a specific increase in resting sodium permeability of the membrane has been suggested as the major mechanism of action on the basis of the reversal of depolarization by a decrease in external sodium concentration (Deguchi & Sakai, 1967; Seyama, 1970). More recently, this mechanism of action has been demonstrated by a more straightforward method in which sodium ions are eliminated from both external and internal media; α-dihydrograyanotoxin II completely fails to depolarize squid axon membranes under this condition (Seyama & Narahashi, 1973).

However, no quantitative measurements have so far been made on the increase in resting sodium permeability of the membrane by any of the depolarizing agents mentioned above. Since the membrane depolarization produced by an increase in resting sodium permeability causes the potassium permeability to increase, the observed change in membrane potential is a sum of the depolarization and repolarization as a result of these ionic permeability changes. Therefore, depolarization of the membrane cannot be taken as an accurate measure of the potency of depolarizing agents in any quantitative manner.

In the present study, the resting sodium permeability was measured by means of voltage clamp techniques on squid giant axons treated with

grayanotoxin I. Such experiments also permitted reliable analyses of the mechanism of grayanotoxin-receptor interaction and that of grayanotoxin-tetrodotoxin antagonism.

METHODS

Materials. Giant axons from the squid, *Loligo pealei*, available at the Marine Biological Laboratory, Woods Hole, Massachusetts, were used. The method of internal perfusion was essentially the same as that described previously (Narahashi & Anderson, 1967).

Solutions. The compositions of the solutions are given in Table 1. External sodium concentration was changed by mixing choline sea-water (solution B) with artificial sea-water (ASW, solution A) at an appropriate ratio. In low sodium high potassium sea-water (solution E), methylsulphate was used as an indiffusible anion through the membrane (Baker, Hodgkin & Shaw, 1962).

Electrophysiological measurements. When appropriate external and internal solutions are chosen from those listed in Table 1, the equilibrium potential for potassium (E_K) becomes equal to that for chloride (E_{Cl}). For example, with solution A outside and solution G inside, E_K and E_{Cl} are -70 mV. With solution E outside and solution I inside, E_K and E_{Cl} are $+12$ mV. The membrane current necessary to hold the membrane potential at these levels was measured under voltage clamp conditions. This current was carried most by sodium ions, for no other freely permeable ions were available either outside or inside of the axon. When grayanotoxin I was applied either externally or internally, there was a large increase in the holding membrane current. Since grayanotoxin I increases the resting sodium permeability selectively, the increment of the current represents a sodium current.

The method of voltage clamp was essentially the same as that described before (Wang, Narahashi & Scuka, 1972; Wu & Narahashi, 1973). The only modification made in the present study was that a 150 μm diameter platinum wire was used instead of a 75 μm platinum wire. This reduced the chance of polarization of the electrode caused by large holding current. In order to further minimize the polarization, the membrane was clamped intermittently for a period of 7 sec at 90 sec intervals with the aid of an electronic switch.

Chemicals. Grayanotoxin I was supplied by Dr Masaiti Yasue of Nagoya City University, Japan. A stock solution was prepared by dissolving toxin in perfusing medium at a concentration of 3×10^{-4} M and kept in a refrigerator. Test solutions were prepared by diluting the stock solution with internal perfusate immediately before use. Tetrodotoxin was purchased from Calbiochem, La Jolla, California, and Tris (tris(hydroxymethyl)aminomethane) from Sigma Chemical Co., St. Louis, Missouri.

Temperature. All experiments were conducted at room temperature of 20° C.

RESULTS

Effect on resting membrane potential

Fig. 2 illustrates the effect of grayanotoxin I on the resting membrane potential. When applied externally, 5×10^{-5} M grayanotoxin I depolarized the membrane from -55 to -15 mV in 46 min. External sodium concentration was then reduced from 449 to 1 mM (solution C of Table 1) in the presence of grayanotoxin I and the membrane was quickly hyper-

TABLE 1. Compositions of external and internal solutions

	External					Internal			
	Artificial sea-water (A) mM	Choline sea-water (B) mM	1 mM Na sea-water (C) mM	Na-free sea-water (D) mM	Low Na high K sea-water (E) mM	Standard internal solution (SIS) (F) mM	Cl internal solution (G) mM	Na-free internal solution (H) mM	High Na low K internal solution (I) mM
Na^+	449	—	1	—	50·8	50	50	—	370
K^+	10	10	10	10	49·3	350	161	400	30
Ca^{2+}	50	50	50	50	50	—	—	—	—
$Choline^+$	—	449	—	—	—	—	—	—	—
Tris	30	30	478	479	30	—	—	—	—
Cl^-	575·8	575·8	379	379·6	116·8	—	35·8	—	191·3
F^-	—	—	—	—	—	50	50	50	50
$Glutamate^-$	—	—	—	—	—	320	95·2	320	128·7
$Methylsulphate^-$	—	—	—	—	100·1	—	—	—	—
$H_2PO^-_4$	—	—	—	—	—	15	15	15	15
Sucrose	—	—	—	—	717·7	333	711	333	333
pH	8·0	8·0	8·0	8·0	8·0	7·3	7·3	7·3	7·3

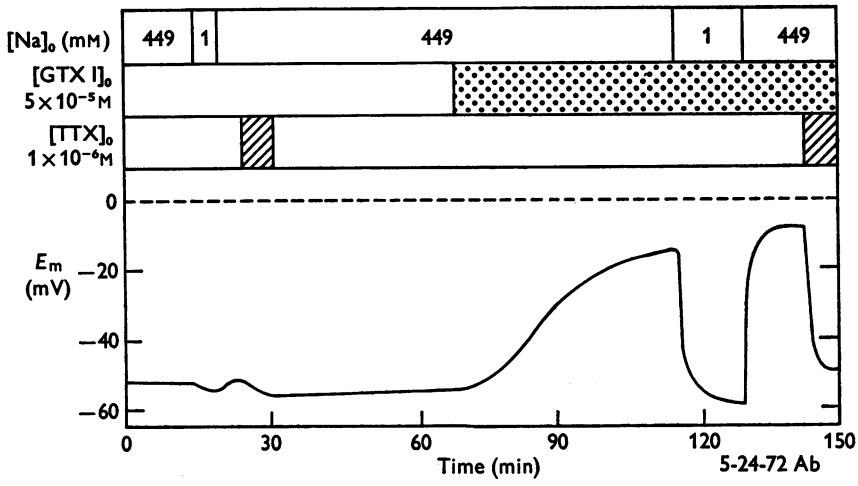

Fig. 2. Changes in resting membrane potential (E_m) of an intact squid axon produced by external application of 5×10^{-5} M grayanotoxin I (GTX I). Decrease of external sodium concentration ([Na]$_o$) to 1 mM hyperpolarizes the membrane in GTX I. Externally applied tetrodotoxin (TTX) also reverses depolarization.

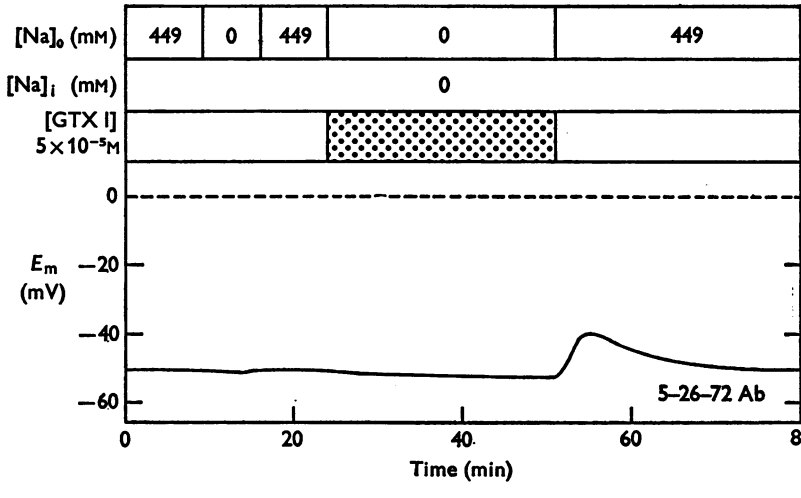

Fig. 3. Effect of externally applied 5×10^{-5} M grayanotoxin I (GTX I) in resting membrane potential (E_m) of an internally perfused squid axon in the absence of sodium ions in both external and internal media. [Na]$_o$ and [Na]$_i$ refer to external and internal sodium concentrations, respectively.

polarized beyond the level observed in 1 mM sodium before application of grayanotoxin I. Readmission of 449 mM sodium produced a membrane depolarization again. Tetrodotoxin at a concentration of 1×10^{-6} M

antagonized the grayanotoxin-induced depolarization, repolarizing the membrane to the original resting potential. This result suggests that the depolarizing action of grayanotoxin I is due to a specific increase in permeability to sodium ions. The depolarization caused by grayanotoxin I was slowly reversed upon washing with toxin-free medium.

If only the sodium permeability were involved in the depolarization caused by grayanotoxin, no change in membrane potential would be expected when both external and internal media were devoid of sodium ions

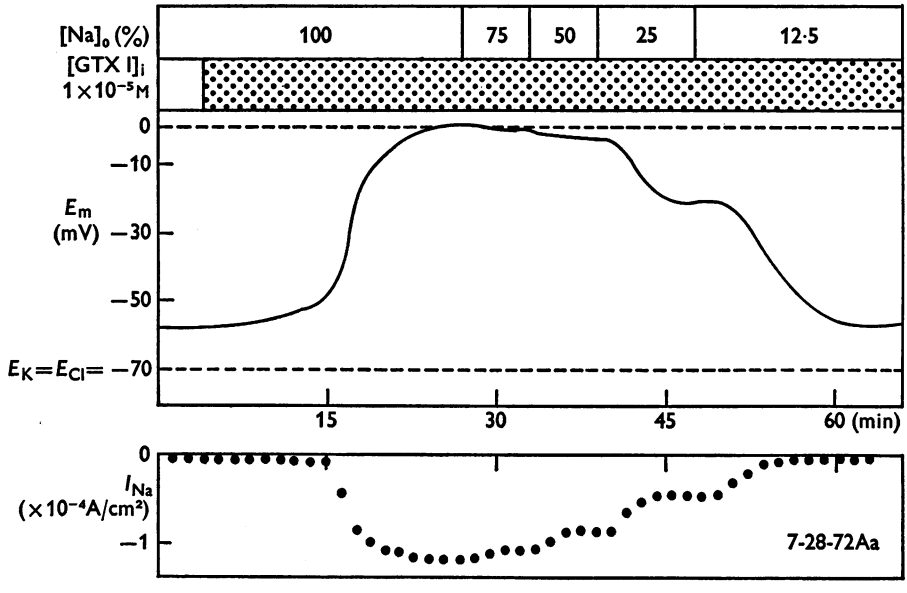

Fig. 4. Changes in resting membrane potential (E_m) and holding membrane current carried by sodium (I_{Na}) by internal perfusion of 1×10^{-5} M grayanotoxin I (GTX I). Current with a minus sign denotes an inward current. External sodium concentration ($[Na]_o$) is 449 mM in normal artificial sea-water (solution A of Table 1), and reduced sodium concentrations are expressed as percentages of that solution. The membrane potential was voltage clamped for 7 sec at 90 sec intervals at − 70 mV which was made equal to the equilibrium potentials for potassium (E_K) and chloride (E_{Cl}) by alteration of the ionic composition of internal perfusate (solution G of Table 1).

(solution D and H of Table 1). Such experiments have indeed demonstrated that this is the case as is shown in Fig. 3. The transient depolarization seen after washing the axon with toxin-free artificial sea-water indicates that grayanotoxin I has bound to the membrane in the absence of sodium. The depolarizing action became apparent when normal sodium concentration was introduced to the external perfusate, but it subsided

as grayanotoxin I was gradually washed out. The result clearly demonstrates that a specific increase in resting permeability to sodium ions is responsible for the depolarization by grananotoxin I.

Measurements of resting sodium conductance and permeability

The membrane current necessary to hold the membrane potential at a level which was made equal to E_K and E_{Cl} was measured under voltage clamp conditions. An example of such an experiment is illustrated in Fig. 4. Artificial sea-water (solution A of Table 1) or mixtures of ASW and choline sea-water (solution B) were used as the external perfusate, and chloride internal solution (solution G) as the internal perfusate. The equilibrium potential for potassium and chloride was -70 mV. The membrane started depolarizing from the control level of -57 mV after internal perfusion of grayanotoxin I at a concentration of 1×10^{-5} M, and attained $+1$ mV in about 20 min. An inward holding current which was carried mostly by sodium ions was increased in magnitude as the membrane depolarization proceeded, and finally reached $-1{\cdot}13 \times 10^{-4}$ A/cm^2. Since the equilibrium potential for sodium (E_{Na}) is $+55$ mV, the resting sodium conductance (g_{Na}) in grayanotoxin I is calculated to be 0·9 mmho/cm^2 from the equation

$$g_{Na} = \frac{I_{Na}}{E_m - E_{Na}}, \tag{1}$$

where E_m is the membrane potential and I_{Na} is sodium current.

The external concentration of sodium was then decreased stepwise in the presence of grayanotoxin I. As it was lowered, the membrane potential was restored toward the initial resting level recorded in 100% sodium, and the inward holding sodium current was decreased in magnitude (Fig. 4).

Since the sodium current cannot satisfactorily be described in terms of sodium conductance when the external sodium concentration is low (Frankenhaeuser, 1960), sodium permeability (P_{Na}) was calculated from the following constant field equation (Goldman, 1943; Hodgkin & Katz, 1949)

$$I_{Na} = P_{Na} \frac{-F^2 E_m}{RT} \frac{[Na]_i - [Na]_o e^{-EF/RT}}{1 - e^{-EF/RT}}, \tag{2}$$

where $[Na]_o$ and $[Na]_i$ are external and internal sodium concentrations, respectively, and F, R and T are the Faraday constant, the gas constant and the absolute temperature, respectively. The mean values for E_m, I_{Na} and P_{Na} are given in Table 2. The sodium permeability was not decreased but rather increased somewhat when external sodium concen-

tration was lowered up to 25% of the normal value, but was decreased in 12·5% sodium.

The sodium currents obtained from the axons treated with grayanotoxin I are plotted as a function of the logarithm of external sodium concentration in Fig. 5. The chain line is drawn by eye. The continuous curve is calculated by the constant field eqn. (2) which assumes a constant

TABLE 2. Membrane sodium current (I_{Na}), sodium permeability (P_{Na}) and membrane potential (E_m) as a function of external sodium concentration ($[Na]_o$) in squid axons perfused internally with 1×10^{-5} M grayanotoxin I

$[Na]_o$ mM	I_{Na} $\times 10^{-6}$ A/cm^2	P_{Na} $\times 10^{-6}$ cm/sec	E_m mV	n
449	$-167{\cdot}4 \pm 18{\cdot}9$	$1{\cdot}31 \pm 0{\cdot}14$	$-2{\cdot}9 \pm 5{\cdot}90$	5
336·8	$-147{\cdot}9 \pm 13{\cdot}6$	$1{\cdot}54 \pm 0{\cdot}14$	$-6{\cdot}8 \pm 5{\cdot}85$	5
224·5	$-110{\cdot}6 \pm 7{\cdot}0$	$1{\cdot}74 \pm 0{\cdot}11$	$-13{\cdot}8 \pm 8{\cdot}44$	5
112·3	$-54{\cdot}4 \pm 7{\cdot}9$	$1{\cdot}74 \pm 0{\cdot}79$	$-32{\cdot}6 \pm 8{\cdot}82$	5
56·1	$-7{\cdot}1 \pm 2{\cdot}8$	$0{\cdot}46 \pm 0{\cdot}19$	$-49{\cdot}7 \pm 3{\cdot}57$	4

Sodium current was measured as the membrane current necessary to voltage clamp the membrane potential at -70 mV which was made equal to the quilibrium potentials for potassium and chloride. Mixtures of solutions A and B of Table 1 were used as external perfusate, and solution G as internal perfusate. E_m in 449 mM $[Na]_o$ before application of grayanotoxin I is $-51{\cdot}8 \pm 1{\cdot}87$ mV.

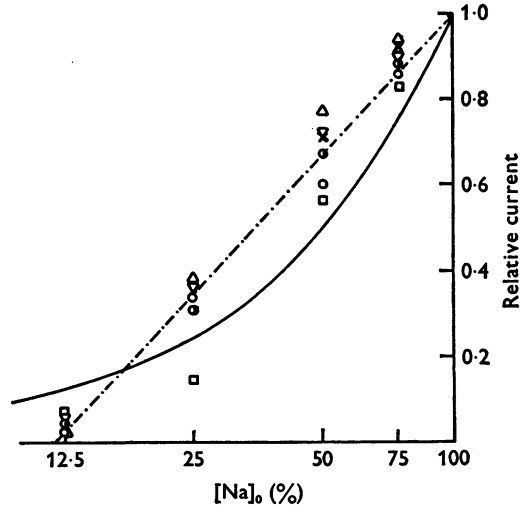

Fig. 5. Resting sodium currents in axons internally perfused with 1×10^{-5} M grayanotoxin I at various external sodium concentrations ($[Na]_o$). Each symbol represents separate series of experiments. Interrupted line is fitted by eye. Continuous curve is drawn by the constant field eqn. (2) for sodium permeability.

sodium permeability. This curve slightly deviates from the measurements, and the possible reasons for this will be discussed later.

The results described in the preceding paragraphs suggest that the sodium channels of the resting nerve membrane are opened up by the action of grayanotoxin I. This leads to an increase in resting sodium conductance. It would then be predicted that resting sodium current could flow in outward direction across the membrane depending on the electrochemical gradient for the sodium. The experiment illustrated in Fig. 6

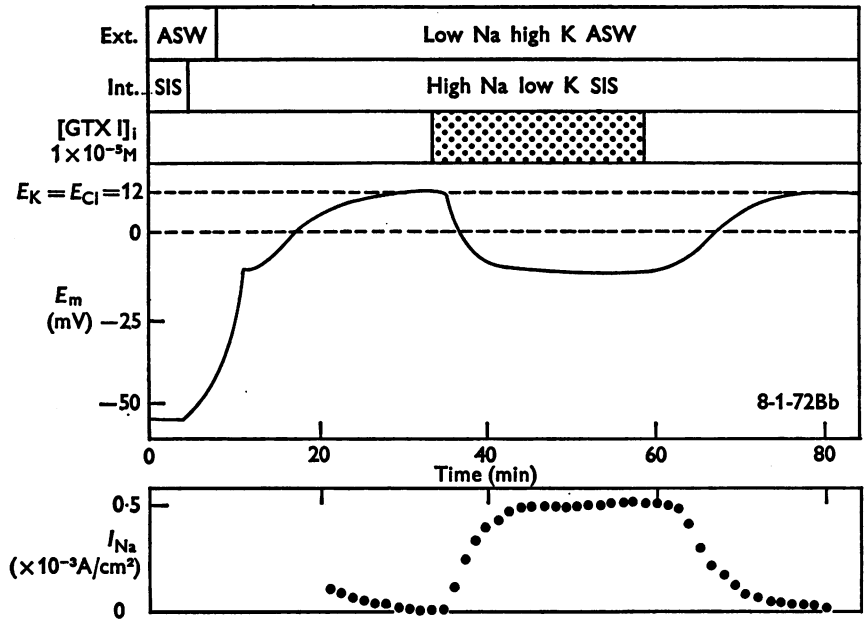

Fig. 6. Changes in resting membrane potential (E_m) and holding membrane current carried by sodium (I_{Na}) by internal perfusion of 1×10^{-5} M grayanotoxin I (GTX I). The similar experiment as that shown in Fig. 4 except that low sodium high potassium sea-water (solution E of Table 1) and high sodium low potassium solution (solution I of Table 1) are used for external and internal perfusates, respectively.

clearly indicates that this is actually the case. In this experiment, low sodium high potassium sea-water (solution E of Table 1) was used as the external perfusate and high sodium low potassium solutions (solution I) as the internal perfusate. The equilibrium potentials for sodium, potassium and chloride are calculated to be -50, $+12$ and $+12$ mV, respectively. In these solutions, the resting membrane potential was reversed and approached E_K which was equal to E_{Cl}. When grayanotoxin was perfused internally at a concentration of 1×10^{-5} M, the membrane was

repolarized toward E_{Na} and attained -12 mV. When the membrane potential was voltage clamped at $+12$ mV during the course of the repolarizing action of grayanotoxin, an outward holding (sodium) current of $0{\cdot}5 \times 10^{-3}$ A/cm^2 flowed, indicating that the resting sodium conductance reached a value of 8 mmho/cm^2. After washing the axon with toxin-free perfusate, the membrane potential returned to $+12$ mV and the outward sodium current was decreased to the level observed before application of the toxin. Numerical data on sodium current and sodium permeability are given in Table 3.

TABLE 3. Membrane sodium current (I_{Na}) and sodium permeability (P_{Na}) measured with a reversed sodium concentration gradient (50·8 mM externally and 370 mM internally) in squid axons internally perfused with 1×10^{-5} grayanotoxin I

Preparation	I_{Na} $\times 10^{-4}$ A/cm^2	P_{Na} $\times 10^{-6}$ cm/sec
8-1-72-Aa	3·72	9·06
8-1-72-Ba	2·50	6·08
8-1-72-Bb	5·01	12·20
8-3-72-Aa	1·27	3·09
8-3-72-Bb	0·81	1·97
Means ± S.E. of mean	2·66 ± 0·80	6·48 ± 1·83

Sodium current was measured as the membrane current necessary to voltage clamp the membrane potential at $+12$ mV which was made equal to the equilibrium potentials for potassium and chloride. Solutions E and I of Table 1 were used as external and internal perfusates, respectively.

Dose–response relationship

Fig. 7 depicts the dose–response relationships of grayanotoxin I in depolarizing the nerve membrane (filled circles) and in increasing the resting sodium current (open circles). Curves were drawn by the following equation which was derived from the Langmuir isotherm on the assumption that drug and receptor interact on a one-to-one stoichiometric basis

$$A = \frac{A_{max}}{1 + K_a/[\text{GTX I}]}, \tag{3}$$

where A is the response as a result of the interaction between grayanotoxin I (GTX I) and the receptor, A_{max} is the maximum response, K_a is the dissociation constant, and [GTX I] is the concentration of grayanotoxin I. The values used to draw curves of Fig. 7 are 41·85 mV and $1{\cdot}342 \times 10^{-4}$ A/cm^2 for A_{max} for depolarization and I_{Na}, respectively, and $1{\cdot}00 \times 10^{-5}$ M and $4{\cdot}12 \times 10^{-5}$ M for K_a for depolarization and I_{Na}, respectively. The measurements of I_{Na} fit the curve very well, indicating that gray anotoxin I reacts with the receptor on a one-to-one stoichiometric basis.

Although the measurements of depolarization also fit the curve, it should be emphasized that the measurements of sodium current give more reliable data in interpreting the dose–response relation than those of depolarization. Since the depolarization produced primarily by an increase in resting sodium permeability following application of grayanotoxin I causes an increase in potassium permeability, which in turn tends

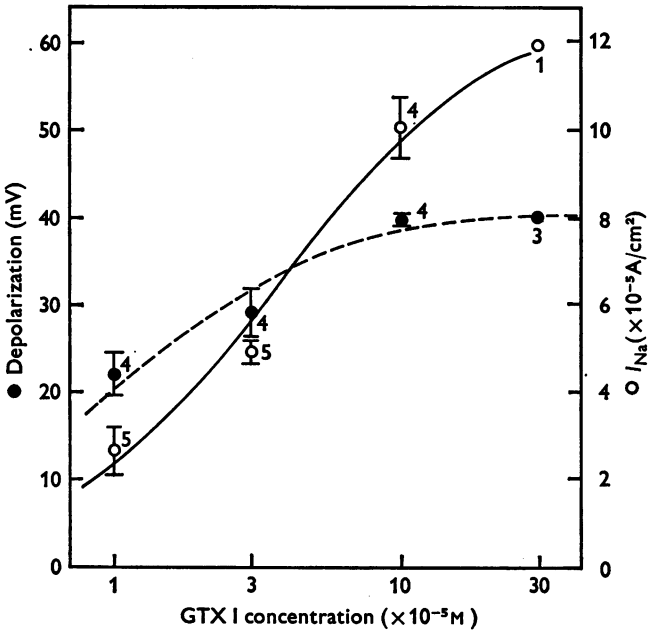

Fig. 7. Dose–response relation for depolarization (filled circles) and increase in sodium current (I_{Na}, open circles) induced by internal application of grayanotoxin I (GTX I). External sodium concentration is reduced to 25% of normal to avoid electrode polarization in high concentrations of GTX I. Lines were fitted by eqn. (3).

to bring the membrane potential back toward the initial resting membrane potential, the potency of grayanotoxin I in depolarizing the membrane is expected to become less as the concentration is increased. This accounts for the large difference between the two curves in Fig. 7.

The one-to-one stoichiometric interaction between grayanotoxin I and receptor was further demonstrated in the analysis of sodium current by means of the Hill's plot in which a unity slope was obtained (Fig. 8).

Tetrodotoxin antagonism

It was shown in a previous section that tetrodotoxin antagonizes the depolarizing action of grayanotoxin I. To clarify the mechanism of tetrodo-

toxin antagonism, measurements were made of sodium current produced by various concentrations of grayanotoxin I applied internally in the presence and absence of 3×10^{-8} M tetrodotoxin externally. The Lineweaver-Burk plots of these data are illustrated in Fig. 9, in which each set of measurements is fitted by a straight line by the least square method. The control line and the tetrodotoxin line intersect the abscissa at points which are not significantly different ($P > 0{\cdot}5$). However, the points where these two lines cross the ordinate are significantly different ($P < 0{\cdot}005$). This leads to the conclusion that tetrodotoxin antagonizes the action of grayanotoxin I in increasing the resting sodium conductance in a non-competitive manner. The apparent dissociation constant for tetrodotoxin was estimated to be $4{\cdot}1 \times 10^{-8}$ M.

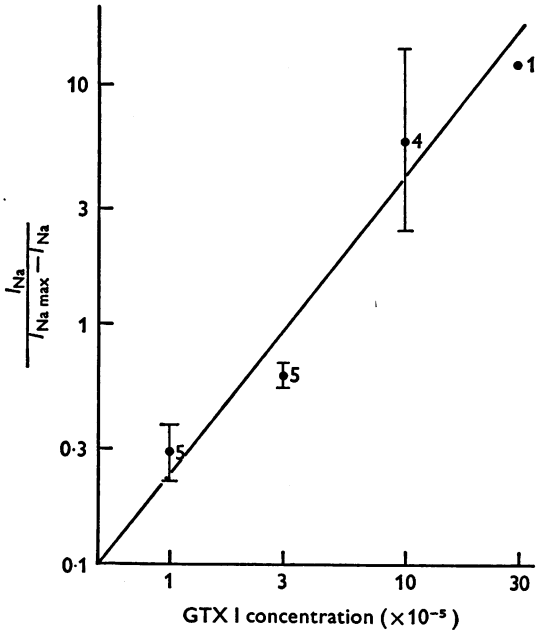

Fig. 8. Hill's plot for the action of internally perfused grayanotoxin I (GTX I) in increasing resting sodium current (I_{Na}). The maximum value for sodium current ($I_{Na\ max}$) was obtained by eqn. (3). Same data as those for Fig. 7.

DISCUSSION

The depolarizing action of grayanotoxin I on squid axon membranes is reversed by a reduction of external sodium concentration or by an application of tetrodotoxin to the external medium. This result agrees with that previously obtained with the skeletal muscles of the frog and rat (Deguchi & Sakai, 1967; Seyama, 1970), and suggests that a specific increase in

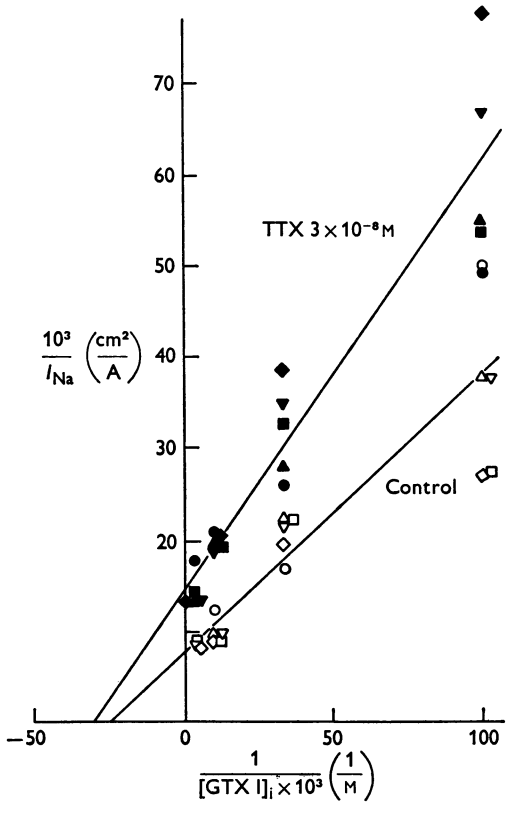

Fig. 9. Lineweaver-Burk plots of increase in sodium current (I_{Na}) caused by internally applied grayanotoxin I (GTX I) and the antagonistic action of tetrodotoxin (TTX) applied externally. $[GTX\ I]_i$ represents internal concentration of GTX I. Each symbol represents separate series of experiments. See text for further explanation. External sodium concentration is reduced to 25% of normal to avoid electrode polarization in high concentrations of GTX I.

resting sodium permeability of the membrane is responsible for the depolarization induced by grayanotoxin I. As in the case of the study of α-dihydrograyanotoxin II on squid axon membranes, this hypothesis has now been demonstrated by the internal perfusion experiment in which grayanotoxin I fails to affect the membrane potential when both external and internal perfusates are devoid of sodium ions.

These results are qualitatively similar to those for batrachotoxin (Narahashi *et al.* 1971), although no quantitative measurement of sodium permeability has been made for batrachotoxin. One important difference is that the depolarization induced by grayanotoxin I is reversible after

washing whereas that by batrachotoxin is not. These two toxins are chemically different also; batrachotoxin is a steroid (Tokuyama, Daly & Witkop, 1969) whereas grayanotoxin I is not (Fig. 1).

In the present study, voltage clamp measurements have proved satisfactory in obtaining more quantitative information as to the increase in resting sodium permeability. The resting sodium permeability of squid giant axons is estimated to be $1{\cdot}5 \times 10^{-8}$ cm/sec from the data of sodium influx (Brinley & Mullins, 1965; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969), and it is increased to $1{\cdot}31 \times 10^{-6}$ cm/sec by 1×10^{-5} M grayanotoxin I (Table 2), indicating about ninetyfold increase.

The resting sodium currents of the axons exposed to grayanotoxin I deviate slightly from the curve predicted from the constant sodium permeability (Fig. 5). This discrepancy may be due to the fact the independence principle (Hodgkin & Huxley, 1952) does not completely hold (see Adelman & Taylor, 1964). An additional possible cause for the discrepancy would be that another ion is involved in the measurement. Since potassium and chloride ions can be excluded, calcium ions are the most likely candidate.

The non-competitive nature of TTX antagonism against the action of grayanotoxin I in increasing sodium permeability deserves further discussion. Grayanotoxin I acts more strongly and quickly with internal application than with external application. The recovery after washing is also faster with internal application. The difference between external and internal applications is more pronounced with grayanotoxin III (I. Seyama & T. Narahashi, unpublished observation). These observations suggest that grayanotoxin I acts from the internal membrane surface. Tetrodotoxin acts only from the external membrane surface (Narahashi, Anderson & Moore, 1966, 1967). Thus, the non-competitive antagonism is to be expected from the point of view of the acting site.

Another important point worthy of note in regard to the non-competitive antagonism is the difference between grayanotoxin and tetrodotoxin in the mechanism of binding to the membrane. The binding of the tritiated tetrodotoxin to nerve membrane preparations shows two phases, one being a saturating binding with a dissociation constant of $3\text{–}10 \times 10^{-9}$ M and the other being a non-saturating binding (Colquhoun, Henderson & Ritchie, 1972). On the contrary, the binding of the tritiated α-dihydrograyanotoxin II to membrane preparation shows only one non-saturating phase (Y. Soeda, R. D. O'Brien, J. Z. Yeh & T. Narahashi, unpublished observation). The apparent dissociation constant for the action of tetrodotoxin in antagonizing the sodium conductance increase caused by grayanotoxin I is estimated to be $4{\cdot}1 \times 10^{-8}$ M, a value one order of mag-

nitude larger than the dissociation constant for the saturating tetrodotoxin binding. Therefore, it seems likely that the saturating binding of tetrodotoxin, for which discrete and very limited membrane sites are assumed (Moore, Narahashi & Shaw, 1967; Hafemann, 1972; Colquhoun *et al.* 1972; Keynes, Ritchie & Rojas, 1971), has no direct bearing on the non-competitive antagonism against the action of grayanotoxin I. It is possible that the non-saturating binding of tetrodotoxin to the membrane is partly responsible for the antagonistic action on grayanotoxin-induced sodium permeability increase and also for the action in decreasing the resting sodium permeability in normal nerve membranes (Narahashi *et al.* 1971; Freeman, 1971; Baker *et al.* 1969).

A corollary of this hypothesis is that there may be two distinct sites or channels for sodium ions; one undergoes a conductance increase upon depolarizing stimulation and is blocked by tetrodotoxin with a dissociation constant of 3×10^{-9} M (Cuervo & Adelman, 1970), and the other is responsible for the resting sodium conductance, is opened up by grayanotoxin, and is sensitive to higher concentrations of tetrodotoxin. Tetrodotoxin antagonizes the action of grayanotoxin I at the latter sodium channel in a non-competitive manner. However, the definitive conclusion about this problem awaits more rigorous experimental analyses.

This study was supported by NIH grant NS 10823, and the experiments were conducted at the Marine Biological Laboratory, Woods Hole, Massachusetts. We wish to express our sincere thanks to Dr Masaiti Yasue for his generous supply of grayanotoxin I samples. Thanks are also due to Mr Edward M. Harris for construction and maintenance of electronic equipment.

REFERENCES

ADELMAN, W. J., Jr. & TAYLOR, R. E. (1964). Effects of replacement of external sodium chloride with sucrose on membrane currents of the squid giant axon. *Biophys. J.* **4**, 451–463.

ALBUQUERQUE, E. X., SEYAMA, I. & NARAHASHI, T. (1973). Characterization of batrachotoxin-induced depolarization of the squid giant axons. *J. Pharmac. exp. Ther.* **184**, 308–314.

BAKER, P. F., BLAUSTEIN, M. P., KEYNES, R. D., MANIL, J., SHAW, T. I. & STEINHARDT, R. A. (1969). The ouabain-sensitive fluxes of sodium and potassium in squid giant axons. *J. Physiol.* **200**, 459–496.

BAKER, P. F., HODGKIN, A. L. & SHAW, T. I. (1962). The effects of changes in internal ionic concentrations on the electrical properties of perfused giant axons. *J. Physiol.* **164**, 355–374.

BRINLEY, F. J. & MULLINS, L. J. (1965). Ion fluxes and transference numbers in squid axons. *J. Neurophysiol.* **28**, 526–544.

COLQUHOUN, D., HENDERSON, R. & RITCHIE, J. M. (1972). The binding of labelled tetrodotoxin to non-myelinated nerve fibres. *J. Physiol.* **227**, 95–126.

CUERVO, L. A. & ADELMAN, JR., W. J. (1970). Equilibrium and kinetic properties of the interaction between tetrodotoxin and the excitable membrane of the squid giant axon. *J. gen. Physiol.* **55**, 309–335.

DEGUCHI, T. & SAKAI, Y. (1967). Sustained after-depolarization in grayanotoxin-treated muscle cell membrane. *J. physiol. Soc. Japan* **29**, 172–173.

FRANKENHAEUSER, B. (1960). Sodium permeability in toad nerve and in squid nerve. *J. Physiol.* **152**, 159–166.

FREEMAN, A. R. (1971). Electrophysiological activity of tetrodotoxin on the resting membrane of the squid giant axon. *Comp. Biochem. Physiol.* **40A**, 71–82.

GOLDMAN, D. E. (1943). Potential, impedance, and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

HAFEMANN, D. R. (1972). Binding of radioactive tetrodotoxin to nerve membrane preparations. *Biochim. biophys. Acta* **266**, 548–556.

HODGKIN, A. L. & HUXLEY, A. F. (1952). Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

KAKISAWA, H., KOZIMA, T., YANAI, M. & NAKANISHI, K. (1965). Stereochemistry of grayanotoxins. *Tetrahedron Lett.* **21**, 3091–3104.

KAKISAWA, H., KURONO, M., NAKANISHI, S. & HIRATA, Y. (1961). Structure of grayanotoxin-I and -III. *Tetrahedron Lett.* **2**, 59–67.

KEYNES, R. D., RITCHIE, J. M. & ROJAS, E. (1971). The binding of tetrodotoxin to nerve membranes. *J. Physiol.* **213**, 235–254.

KOZIMA, T., NAKANISHI, K., YANAI, M. & KAKISAWA, H. (1964). The full stereochemistry of the grayanotoxins and a novel rearrangement. *Tetrahedron Lett.* **21**, 1329–1336.

KUMAZAWA, Z. & IRIYE, R. (1970). Stereochemistry of grayanotoxin-II. *Tetrahedron Lett.* **12**, 927–930.

MATSUMOTO, T. & WATANABE, M. (1968). Stereochemistry of grayanotoxin. *Tetrahedron Lett.* **57**, 6019–6022.

MOORE, J. W., NARAHASHI, T. & SHAW, T. I. (1967). An upper limit to the number of sodium channels in nerve membrane? *J. Physiol.* **188**, 99–105.

NARAHASHI, T., ALBUQUERQUE, E. X. & DEGUCHI, T. (1971). Effects of batrachotoxin on membrane potential and conductance of squid giant axons. *J. gen. Physiol.* **58**, 54–70.

NARAHASHI, T. & ANDERSON, N. C. (1967). Mechanism of excitation block by the insecticide allethrin applied externally and internally to squid giant axons. *Toxic appl. Pharmac.* **10**, 529–547.

NARAHASHI, T., ANDERSON, N. C. & MOORE, J. W. (1966). Tetrodotoxin does not block excitation from inside the nerve membrane. *Science, N.Y.* **153**, 765–767.

NARAHASHI, T., ANDERSON, N. C. & MOORE, J. W. (1967). Comparison of tetrodotoxin and procaine in internally perfused squid giant axons. *J. gen. Physiol.* **50**, 1413–1428.

OHTA, M., NARAHASHI, T. & KEELER, R. F. (1973). Effects of veratrum alkaloids on membrane potential and conductance of squid and crayfish giant axons. *J. Pharmac. exp. Ther.* **184**, 143–154.

SEYAMA, I. (1970). Effect of grayanotoxin I on the electrical properties of rat skeletal muscle fibers. *Jap. J. Physiol.* **20**, 381–393.

SEYAMA, I. & NARAHASHI, T. (1973). Increase in sodium permeability of squid axon membranes by α-dihydrograyanotoxin II. *J. Pharmac. exp. Ther.* **184**, 299–307.

TOKUYAMA, T., DALY, J. & WITKOP, B. (1969). The structure of batrachotoxin, a steroidal alkaloid from the Colombian arrow poison frog, *Phyllobates aurotaenia*, and partial synthesis of batrachotoxin and its analogs and homologs. *J. Am. chem. Soc.* **91**, 3931–3938.

ULBRICHT, W. (1969). The effect of veratridine on excitable membranes of nerve and muscle. *Ergebn. Physiol.* **61**, 17–71.

WANG, C. M., NARAHASHI, T. & SCUKA, M., (1972). Mechanism of negative temperature coefficient of nerve blocking action of allethrin. *J. Pharmac. exp. Ther.* **182**, 442–453.

WU, C. H. & NARAHASHI, T. (1973). Mechanism of action of propranolol on squid axon membranes. *J. Pharmac. exp. Ther.* **184**, 155–162.