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Cation Permeability Ratios of Sodium Channels in Normal and Grayanotoxin-Treated Squid Axon Membranes

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Summary. Permeabilities of squid axon membranes to various cations at rest and during activity have been measured by voltage clamp before and during internal perfusion of 4×10^{-5} M grayanotoxin I. The resting sodium and potassium permeabilities were estimated to be 6.85×10^{-8} cm/sec and 2.84×10^{-6} cm/sec, respectively. Grayanotoxin I increased the resting sodium permeability to 7.38×10^{-7} cm/sec representing an 11-fold increase. The potassium permeability was increased only by a factor of 1.24. The resting permeability ratios as estimated by the voltage clamp method before application of grayanotoxin I were Na (1): Li (0.83): formamidine (1.34): guanidine (1.49): Cs (0.87): methylguanidine (0.86): methylamine (0.78). Grayanotoxin I did not drastically change the resting permeability ratios with a result of Na (1): Li (0.95): formamidine (1.27): guanidine (1.16): Cs (0.47): methylguanidine (0.72): methylamine (0.46). The membrane potential method gave essentially the same resting permeability ratios before and during application of grayanotoxin I if corrections were made for permeability to choline as the cation substitute and for changes in potassium permeability caused by test cations. The permeability ratio choline/Na was estimated to be 0.72 by the voltage clamp method and 0.65 by the membrane potential method. Grayanotoxin I decreased the ratio to 0.43. The permeability ratios during peak transient current were estimated to be Na (1): Li (1.12): formamidine (0.20): guanidine (0.20): Cs (0.085): methylguanidine (0.061): methylamine (0.036). Thus the sodium channels for the peak current are much more selective to cations than the resting sodium channels. It appears that the resting sodium channels in normal and grayanotoxin I-treated axons are operationally different from the sodium channels that undergo a conductance increase upon stimulation.

It has been well established that nerve membranes undergo permeability increases to a variety of cations during excitation. Peak transient currents associated with step depolarizations in voltage clamp axons are carried mostly by sodium ions under normal conditions, but they can be

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carried by other inorganic and organic cations to varying extents. Thus the inorganic cation permeability ratio during the peak transient current has been estimated to be Na/Li/K/Rb/Cs = 1 : 1.1 : 0.083 : 0.025 : 0.016 in squid axons (Chandler & Meves, 1965), and 1:0.93:0.086:0.012:0.013 in frog nodes of Ranvier (Hille, 1972). The nodal membrane is also permeable to a variety of organic cations during activity (Hille, 1971). For example, hydroxylamine can penetrate the membrane almost equally to sodium; formamidine and guanidine are much less permeant, and methylamine is almost impermeant.

However, the nature of ionic permeabilities in resting conditions largely remains to be seen. The nerve membrane at rest is permeable to various cations, and the ratio $K/Rb/NH_4/Cs/Na/Li = 1:0.71:0.25:0.18:<0.08:<0.08$ has been obtained with squid giant axons (Hagiwara, Eaton, Stuart & Rosenthal, 1972). No data are available for the resting permeability to various organic cations.

Recently, certain toxins and alkaloids have been found to increase selectively the resting nerve membrane permeability to sodium, thereby causing a sizable depolarization. Among these are batrachotoxin (Narahashi, Deguchi & Albuquerque, 1971; Narahashi, Albuquerque & Deguchi, 1971; Albuquerque, Seyama & Narahashi, 1973), veratridine (Ohta, Narahashi, Keeler, 1973; Ulbricht, 1969 *a*, *b*), α-dihydrograyanotoxin II (Seyama & Narahashi, 1973), and grayanotoxin I (Narahashi & Seyama, 1974). In spite of the large reversible increase in resting sodium permeability caused by grayanotoxins, the sodium permeability still undergoes a normal increase upon stimulation provided that the membrane potential is restored to the original level by a hyperpolarizing current. This and several other observations raise a question as to whether the passive sodium movements across the membrane at rest and during activity occur through the same ionic channels.

The present study has been undertaken in order to characterize the cation permeability of sodium channels. Permeabilities to a variety of cations at rest and during activity were measured in normal squid giant axons and in axons treated with grayanotoxin I, since the toxin selectively increases the resting sodium permeability, thereby providing a chance to characterize the toxin-induced component of resting sodium permeability. It was found that the cation permeability ratios at rest are distinctly different from those during activity, and that grayanotoxin I does not significantly alter either of the cation permeability ratios.

Preliminary accounts of this study have been reported (Hironaka & Narahashi, 1975, 1976).

Materials and Methods

Materials and Internal Perfusion

Giant axons of 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

For measurements of resting permeabilities, artificial sea water of the following composition was used as the external bathing medium (mM): Na^+, 450; K^+, 10; Ca^{++}, 50; Cl^-, 565.6; tris(hydroxymethyl)aminomethane (Tris), 10; at final pH 8.0. Internal perfusate contained (mM): Na^+, 50; K^+, 161; Cl^-, 35.8; F^-, 145.2; $H_2PO_4^-$, 14.5; sucrose, 711; final pH 7.3. To prepare external test cation solutions, Na was replaced with test cations. When the external sodium or test cation concentration was to be reduced, choline was used as the substitute.

For measurements of ionic permeability ratios during peak transient current, an artificial sea water in which K was replaced by Na was used as the external perfusate. The internal perfusate for normal axons contained (mM): Na^+, 50; K^+, 350; glutamate$^-$, 320; F^-, 50; $H_2PO_4^-$, 15; sucrose, 333; final pH 7.3. The internal perfusate for the axons internally treated with grayanotoxin I contained (mM): Na^+, 59.1; K^+, 311.1; Cl^-, 59.1; glutamate$^-$, 281.1; $H_2PO_4^-$, 15; sucrose, 370; final pH 7.3.

Electrophysiological Measurements

Resting permeability. The nerve membrane permeability to various cations at resting condition was measured by two methods, i.e., voltage clamp method and membrane potential method. The voltage clamp method was based on the measurements of resting ionic currents carried by test cations. The membrane permeabilities to the test cations were calculated by the constant field equation as described later. The method of voltage clamp was essentially the same as that described previously (Narahashi & Seyama, 1974). In the present study, however, a platinum/iridium wire (90 : 10) with a diameter of 100 μm was used instead of a 150 μm platinum wire as the internal current electrode. The fine platinum wire inserted in the previous study to reduce high frequency impedance was omitted. The nerve membrane was voltage clamped at the resting potential and step hyperpolarizations of 40 msec duration were applied to bring the membrane potential to −70, −90, −110 and −130 mV. The membrane currents associated with the hyperpolarizations were measured at 15 and 25 msec from the beginning of the pulse. Since the equilibrium potentials for chloride (E_{Cl}) and potassium (E_K) were both −70 mV with the internal and external solutions used, the membrane currents at −70 mV were carried mostly by sodium or test cations (Narahashi & Seyama, 1974).

Measurements of resting ionic permeabilities by the membrane potential method were based on application of the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$E_m = \frac{RT}{F} \ln \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o} \tag{1}$$

where E_m refers to the membrane potential, P refers to the permeability of ion in the subscript, [ ] refers to the concentration (strictly speaking, the activity) of the ion in outside (o) or inside (i) of the axon, and R, T and F refer to the gas constant, absolute temperature and Faraday

constant, respectively. When the external sodium concentration was to be reduced, sodium was partially replaced with choline. Similar measurements of the membrane potential were repeated with varying concentrations of a test cation in place of sodium. The concentrations of all ions except for sodium, choline and test cations were kept constant. Thus, at the sodium and test cation (X) concentrations where the same membrane potential is obtained, the following relations are deduced from Eq. (1), provided that permeabilities to other ions are kept constant (Hagiwara, Toyama & Hayashi, 1971):

$$\frac{P_X}{P_{\mathrm{Na}}} = \frac{[\mathrm{Na}]_o}{[X]_o}. \tag{2}$$

The recording system of the membrane potential was essentially the same as that used for voltage clamp. A glass capillary of 75 or 100 μm diameter filled with 0.6 M KCl solution was used as the internal potential electrode, and was connected to a high input impedance preamplifier via an Ag-AgCl wire. The external reference electrode was similar to the internal potential electrode, but was filled with 2 M KCl-agar. The zero potential was set with the internal and external electrode tips immersed in internal and external perfusates, respectively, which were electrically connected by a 3 M KCl-agar bridge. The changes in junction potential associated with replacing external sodium with test cations were less than 0.5 mV for which no correction was made. The change in membrane potential was measured at a steady state which was attained in 4 to 5 min after changing the external solution. Since the membrane potential did not completely return to the original level but slightly decreased after application of a series of test cations, the measurements were corrected for deterioration as described later.

Permeability during activity. The cation permeability ratio during peak transient current was estimated by measurements of the reversal potential under voltage clamp conditions by the method similar to that used by Chandler and Meves (1965). The method of voltage clamp was the same as described in the preceding section except that a bare platinum wire of 25 μm in diameter was inserted in the internal capillary electrode to reduce high frequency impedance. The membrane potential was held at −80 mV and step depolarizing pulses, varying by 1 mV steps near the reversal potential for peak transient current, were applied. The membrane potential at which no inward or outward peak current flowed was taken as the reversal potential (Goldman and Binstock, 1969).

The ratio of test cation permeability to sodium permeability during peak transient current was calculated from the equation

$$\frac{P_X}{P_{\mathrm{Na}}} = \frac{[\mathrm{Na}]_o}{[X]_o} e^{F(E_{rX} - E_{r\,\mathrm{Na}})/RT} \tag{3}$$

where E_{rX} and $E_{r\,\mathrm{Na}}$ represent the reversal potentials in test cation and sodium, respectively.

Chemicals

Grayanotoxin I (GTX I) is one of the toxic principles obtained from the leaves of various plants that belong to the family Ericaceae. It is a heterocyclic diterpane, and the stereochemical structure has been completely identified (*see* Narahashi, 1974). Grayanotoxin I was dissolved in ethanol at a concentration of 1.6×10^{-2} M to make up a stock solution which was kept refrigerated. The stock solution was diluted with internal perfusate to give a final concentration of 4×10^{-5} M immediately before use. Thus, the concentration of ethanol in test solutions was 0.25% (v/v) and had no effect on the electrical parameters measured.

Temperature

Measurements of resting cation permeabilities by the voltage clamp method were performed at 20–22 °C, and those by the membrane potential method at 18 °C. Cation permeability ratios during peak transient current were measured at 9 °C.

Results

Resting Ionic Permeabilities as Measured by Voltage Clamp Method

Sodium and potassium permeabilities of resting membrane. The membrane potential was clamped at the resting level and a step hyperpolarization was applied to bring the membrane potential to −70 mV, which was equal to the potassium and chloride equilibrium potentials under the present experimental conditions (*see* Materials and Methods). Fig. 1 illustrates the records of membrane potential and membrane current before and after internal application of GTX I at a concentration of 4×10^{-5} M. Since the steady-state membrane current is carried mostly by sodium

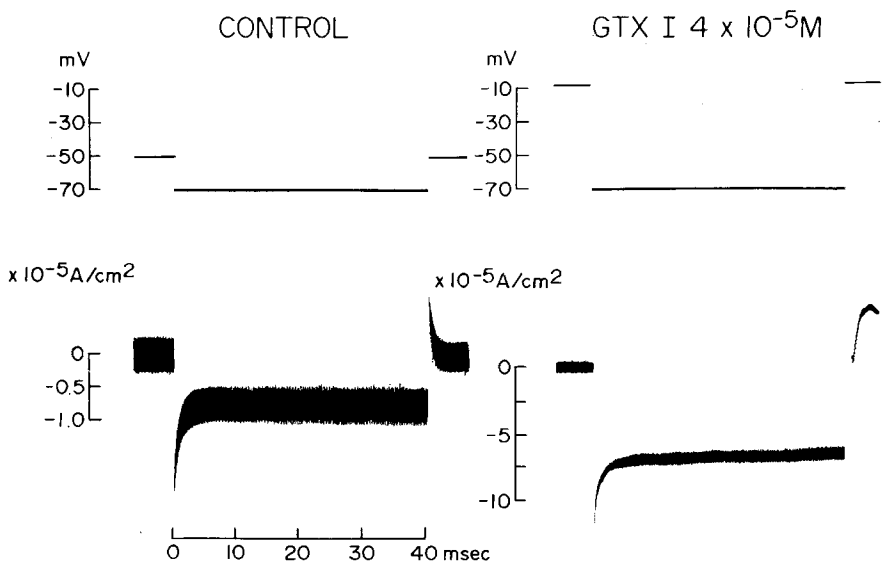


Fig. 1. Membrane current associated with a step hyperpolarization from the resting membrane potential to −70 mV which was made equal to the equilibrium potentials for potassium and chloride by adjusting external and internal ionic compositions. The membrane current under this condition is carried largely by sodium ions. Note that grayanotoxin I (GTX I) greatly depolarized the membrane and increased the membrane current. Negative sign denotes an inward current

ions under this experimental condition, the sodium permeability can be calculated by the constant field equation (Goldman, 1943; Hodgkin & Katz, 1949),

$$P_{Na} = -I_{Na} \frac{RT}{E_m F^2} \frac{1 - e^{E_m F/RT}}{[Na]_o - [Na]_i e^{E_m F/RT}}, \quad (4)$$

where I_{Na} refers to sodium current. The sodium permeability was estimated to be $6.85 \pm 0.65 \times 10^{-8}$ cm/sec on average (20 axons).

During internal perfusion with GTX I at a concentration of 4×10^{-5} M, the membrane was greatly depolarized from the control value of -50 mV to -8 mV, and the membrane current associated with a step hyperpolarization to -70 mV was substantially increased (Fig. 1). The sodium permeability in GTX I was estimated to be $73.8 \pm 3.2 \times 10^{-8}$ cm/sec on average (n = 20) representing an 11-fold increase in P_{Na}.

The next step was to estimate the resting potassium permeability in control axons. The membrane was hyperpolarized in a double step from the resting level to -70 mV first and then to -90 mV. The membrane current associated with the hyperpolarization to -90 mV was carried not only by sodium but also by potassium and chloride, because the potential level was away from the potassium and chloride equilibrium potentials which were made equal to -70 mV. Assuming that P_{Na} was kept constant at -70 mV and -90 mV, I_{Na} at -90 mV could be calculated by Eq. (4). The chloride permeability is only a few percent of P_K (Baker, Hodgkin & Shaw, 1962; Baker, Hodgkin & Meves, 1964), and therefore can be omitted from the calculation. Thus the potassium current was calculated by subtracting the sodium current obtained above from the total membrane current at -90 mV. The potassium permeability was then calculated by the constant field equation for potassium similar to Eq. (4), and amounted to $2.84 \pm 0.40 \times 10^{-6}$ cm/sec on average (n = 20). The average P_{Na}/P_K ratio was estimated to be 0.031 ± 0.003.

The resting membrane potential was calculated by incorporating the P_{Na}/P_K value obtained for each axon into the following equation in which the ratio P_{Cl}/P_{Na} was assumed to be 0.65:

$$E_m = \frac{RT}{F} \ln \frac{[K]_o + \frac{P_{Na}}{P_K}[Na]_o + \frac{P_{Cl}}{P_K}[Cl]_i}{[K]_i + \frac{P_{Na}}{P_K}[Na]_i + \frac{P_{Cl}}{P_K}[Cl]_o}. \quad (5)$$

The calculated membrane potentials are plotted against the observed membrane potential in Fig. 2. The data fall on a unity slope. The average

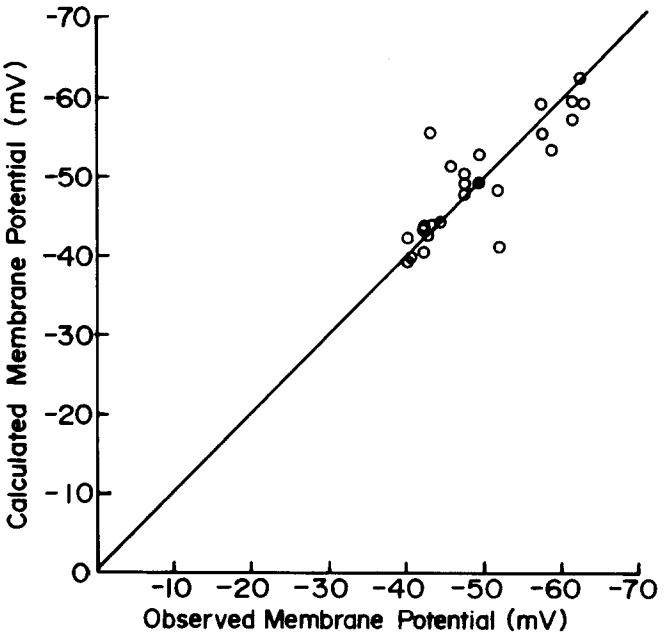


Fig. 2. Calculated resting membrane potentials plotted as a function of the observed resting membrane potentials in normal external bathing solution. The calculations were made using the constant field Eq. (5). The ratio P_{Na}/P_K was measured from the membrane currents at -70 and -90 mV by using Eq. (4). *See* text for further explanation. Filled circles represent the average values, and solid line a slope of unity

membrane potential was estimated to be -49.0 ± 1.6 mV, and the average P_{Na}/P_K and P_{Cl}/P_K ratio 0.031 ± 0.003 and 0.021 ± 0.002, respectively (n = 24). These results justify the use of the constant field equation, and therefore predict that the ionic permeability measurements by the voltage clamp and membrane potential methods would give reasonable agreement.

Change in resting sodium and potassium permeabilities with time. Since the experiments for measurements of membrane permeabilities to various test cations were performed over a period of about 150 min in each axon, changes in sodium permeability with time was measured and used to correct the permeability ratio P_X/P_{Na}. The sodium permeability measured at various times after starting experiment ($P_{Na(t)}$) is plotted as the ratio to its initial value ($P_{Na(o)}$) against the time in Fig. 3. The sodium permeability increased linearly with time at a rate of 77% per hr.

The data presented in Fig. 3 were also used to correct the membrane potential. Since the membrane potential is a logarithmic function of the sodium permeability, the linear increase in sodium permeability indicates that depolarization occurs in a logarithmic manner with time.

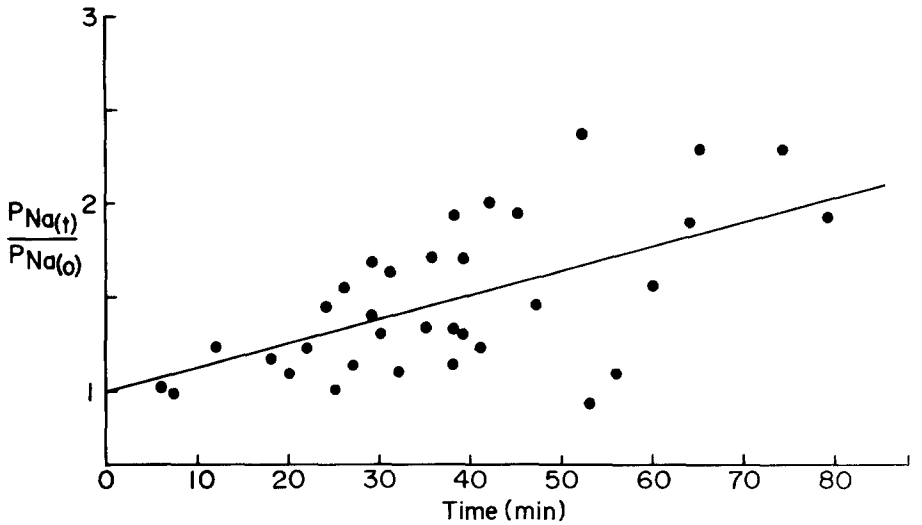


Fig. 3. Sodium permeability (P_{Na}) measured at various times after starting experiments. The values for P_{Na} were measured before and after experiments with test cations in each axon from the membrane current at -70 mV using Eq. (4). The ordinate indicates the P_{Na} value at time t relative to that at zero time

Potassium permeability underwent little or no change with the progress of time.

Resting permeability ratios test cations/sodium. After measurements of the membrane current associated with step hyperpolarization to -70 mV, a test cation was substituted for sodium in external medium and the measurement was repeated. The membrane current thus measured in the test solution consists of an inward current carried by the test cation and an outward current carried by sodium. The permeability ratio P_X/P_{Na} can be calculated by the following equation which is derived from the constant field Eq. (4):

$$\frac{P_X}{P_{Na}} = \frac{1}{[X]_o} \left\{ \frac{I'_X}{I_{Na}} ([Na]_o - [Na]_i \, e^{E_m F/RT}) + [Na]_i \, e^{E_m F/RT} \right\} \qquad (6)$$

where I'_X refers to the membrane current at -70 mV in the test solution. The observed ratio P_X/P_{Na} was corrected for the small linear increase in P_{Na} with time (Fig. 3).

The relative permeabilities of various test cations are given in Table 1. Formamidine and guanidine were more permeant than sodium with the P_X/P_{Na} ratios of 1.34 and 1.49, respectively. Lithium, cesium, methylguanidine and methylamine were less permeant than sodium with the

Table 1. Resting cation permeabilities (P_X) relative to sodium permeability (P_{Na}) before and during internal perfusion of 4×10^{-5} M grayanotoxin I (GTX I) as measured by voltage clamp method

Cation	P_X/P_{Na}	
	Control	GTX I
Na	1.00	1.00
Li	0.83±0.08 (6)	0.95±0.06 (6)
Formamidine	1.34±0.07 (9)	1.27±0.05 (10)
Guanidine	1.49±0.07 (11)	1.16±0.07 (11)
Cs	0.87±0.03 (7)	0.47±0.05 (9)
Methylguanidine	0.86±0.08 (7)	0.72±0.10 (8)
Methylamine	0.78±0.05 (10)	0.46±0.07 (11)

Data are given in the mean ±SEM with the number of measurements in parentheses.

P_X/P_{Na} ratios of 0.83, 0.87, 0.86 and 0.78, respectively. However, the difference between the most permeant and the least permeant cations was relatively small, the permeability ratio of guanidine to methylamine being 1.91.

The permeability ratio P_X/P_{Na} was also measured during internal perfusion of GTX I at a concentration of 4×10^{-5} M (Table 1). Although the absolute values for cation permeabilities were markedly increased by GTX I, the ratios P_X/P_{Na} were not drastically altered. Formamidine and guanidine were more permeant than sodium as in the control. The ratio P_{Li}/P_{Na} increased slightly from 0.83 to 0.95 in GTX I, and the methylguanidine permeability relative to the sodium permeability slightly decreased from 0.86 to 0.72. The cesium and methylamine permeabilities relative to the sodium permeability decreased in GTX I from 0.87 to 0.47 and from 0.78 to 0.46, respectively.

Resting potassium permeability in test cation solutions. Resting potassium permeability was calculated from the membrane current measured at −90 mV as described before. The measurements were made before, during, and after application of a test cation solution, and changes in P_K are given in Table 2. The potassium permeability was not greatly affected by substitution of lithium, formamidine or methylamine for external sodium, the ratios of P_K in the test cations to that in sodium being 1.07, 0.97 and 0.93, respectively. However, cesium, guanidine and methylguanidine decreased P_K, the ratios of P_X in the test cations to that in sodium being 0.56, 0.72 and 0.67, respectively. In all cases, the recovery of P_K after washing the axon with normal sodium solution was near complete as can be seen from near unity values for the ratios of P_K after to P_K before exposure to test cations (Table 2).

Table 2. Changes in resting potassium permeability (P_K) by test cations (X) in a value relative to P_K in sodium solution

Cation	$\frac{P_K \text{ in } X}{P_K \text{ in Na}}$	$\frac{P_K \text{ in Na after } X}{P_K \text{ in Na}}$	N
Na	1.00	1.00	
Li	1.07±0.18	1.05±0.20	6
Formamidine	0.97±0.09	0.94±0.14	6
Guanidine	0.72±0.16	1.14±0.25	7
Cs	0.56±0.08	0.99±0.20	6
Methylguanidine	0.67±0.11	1.05±0.20	6
Methylamine	0.93±0.07	1.03±0.20	6
Choline	0.92±0.19	0.90±0.08	4

Data are given in the mean ±SEM

It should be noted that the observed decrease in P_K in cesium and methylguanidine solutions is consistent with the observation that both cations decreased the membrane potential with an increase in concentration to a greater extent than sodium (Fig. 5*A*) despite their lower permeabilities than sodium (Table 1).

Resting potassium permeability in the axon treated with GTX I (4×10^{-5} M) was measured by the method similar to that for the control axons. However, sodium-free choline solution was used instead of sodium solution, since no negative conductance was observed in the choline solution at the membrane potentials ranging from −70 mV to −90 mV where potassium permeability was measured.

The potassium permeability was increased by GTX I by a factor of 1.24±0.13 (4 axons) attaining a value of 3.52×10^{-6} cm/sec. Since the sodium permeability in GTX I is increased to 0.74×10^{-6} cm/sec as described before, the nerve membrane treated with GTX I becomes much less selective to sodium and potassium at resting conditions.

Resting Ionic Permeabilities as Measured by Membrane Potential Method

Fig. 4 illustrates an example of an experiment to measure the membrane potentials in sodium and formamidine solutions before and during internal perfusion of 4×10^{-5} M GTX I. The membrane potential was measured in 450 mM Na and in various concentrations of formamidine. The mean values for the membrane potentials are plotted as a function of the external sodium and test cation concentrations in Fig. 5. In both

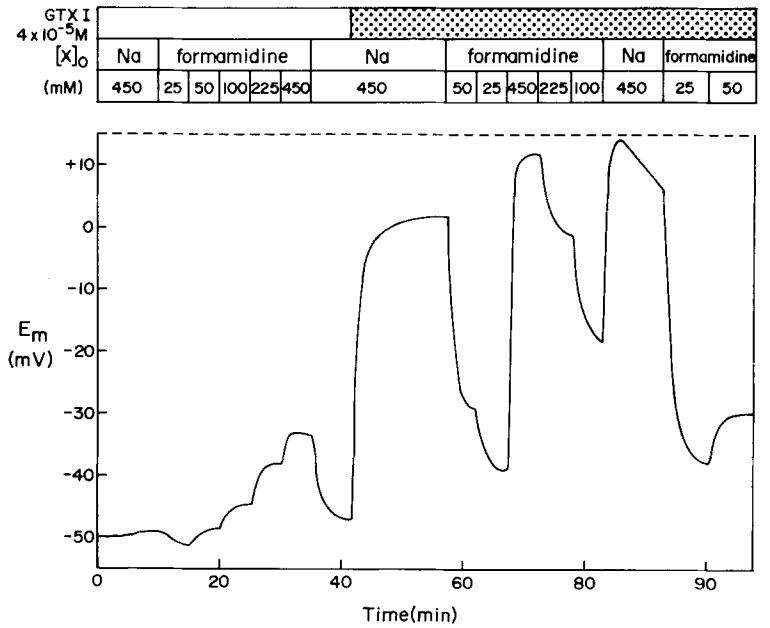
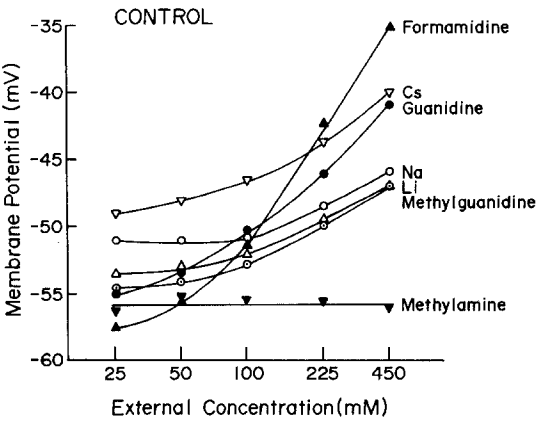


Fig. 4. Resting membrane potentials in normal sodium-containing external solution and in solutions with various concentrations of formamidine without sodium before and during internal perfusion of 4×10^{-5} M grayanotoxin I (GTX I). In solutions with formamidine at concentrations of less than 450 mM, equimolar concentrations of choline were substituted for formamidine

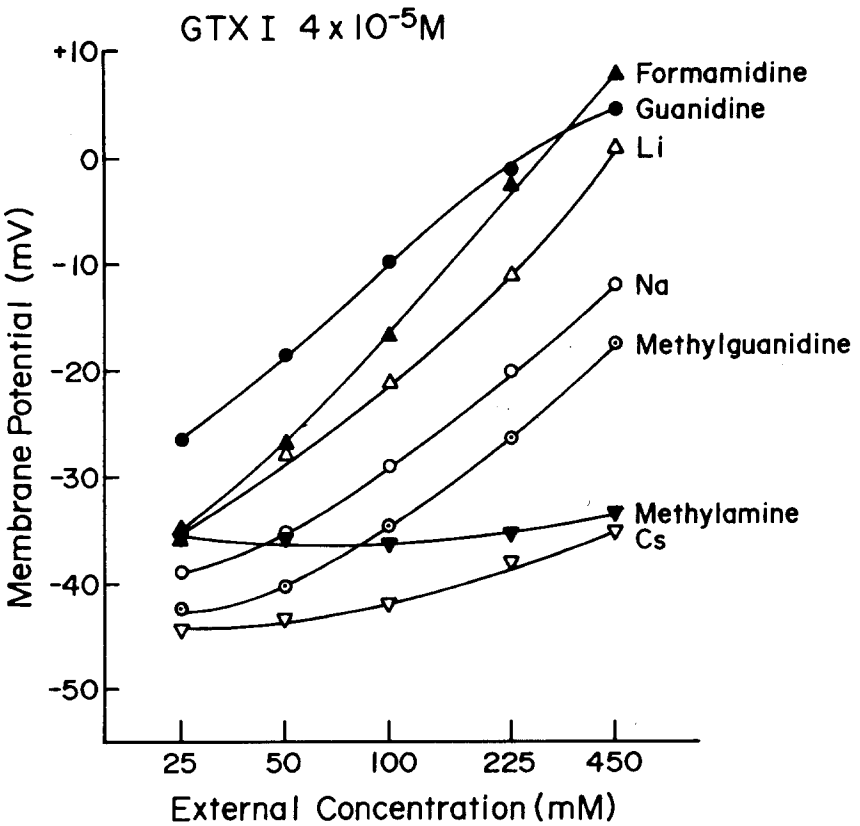
control and GTX I experiments, the membrane was hyperpolarized when the external cation concentration was decreased with the exception of methylamine in which little or no membrane potential change occurred.

The cation permeability ratios P_X/P_{Na} were calculated by Eq. (2) using the data presented in Fig. 5, and are given in Table 3. Before application of GTX I (control, before correction), the permeability ratios for cesium, formamidine, guanidine and methylguanidine are much greater than those obtained by the voltage clamp method (Table 1). During application of GTX I, there are also discrepancies between the ratios in Table 3 and those in Table 1; the permeability ratios for lithium, formamidine and guanidine are greater with the membrane potential method than with the voltage clamp method, whereas those for cesium and methylguanidine are smaller. It will be shown that this is due to the neglect of incorporating choline and potassium permeability changes into the constant field equation.

Resting potassium permeability change caused by choline. Resting potassium permeability was measured by the voltage clamp method in



a



b

Fig. 5. Resting membrane potential as a function of sodium and test cation concentrations in the external perfusate before and during internal perfusion of 4×10^{-5} M grayanotoxin I (GTX I). Solid lines were drawn by eye. Each point represents the mean of 7 axons

Table 3. Resting cation permeabilities (P_X) relative to sodium permeability (P_{Na}) before and during internal perfusion of 4×10^{-5} M grayanotoxin I (GTX I) as measured by the membrane potential method

	Before corrections		After corrections	
	Control	GTX I	Control	GTX I
Na	1.00	1.00	1.00	1.00
Li	1.15	1.22	1.05	1.12
Formamidine	4.95	2.74	2.81	1.30
Guanidine	5.00	1.89	1.21	1.30
Cs	4.79	0.12	0.92	0.29
Methylguanidine	3.13	0.26	0.96	0.41
Methylamine	–	–	0.64	0.48

Data are given in the permeability ratios from the mean membrane potentials of 7 axons for each cation except for Li, for which 6 axons were used.

normal sodium solution and in solutions in which choline was substituted for sodium. The potassium permeability in choline solution relative to that in sodium solution was estimated to be 0.93 ± 0.07 in four axons (Table 2). Thus the change in potassium permeability caused by choline substitution is negligible.

Resting choline permeability. Resting choline permeability was measured by the voltage clamp method. The permeability ratio of choline to sodium was relatively high, and is estimated to be 0.72 ± 0.12 from four normal axons. During internal perfusion of 4×10^{-5} M GTX I, the ratio decreased to 59% of the control to give a value of 0.43 ± 0.05 (n = 4).

Neglect of including the choline permeability term in Eq. (5) accounts for a large deviation of the calculated curve from the observations as is shown in Fig. 6, in which the membrane potential is plotted as a function of the external sodium concentration. The ratios P_{Na}/P_K and P_{Cl}/P_K were estimated to be 0.031 and 0.021, respectively, in a previous section, and these values were used for the calculation. Thus Eq. (5) is modified by including the choline term to give

$$E_m = \frac{RT}{F} \ln \frac{[K]_o + \frac{P_{Na}}{P_K}[Na]_o + \frac{P_{Ch}}{P_K}[Ch]_o + \frac{P_{Cl}}{P_K}[Cl]_i}{[K]_i + \frac{P_{Na}}{P_K}[Na]_i + \frac{P_{Cl}}{P_K}[Cl]_o} \tag{7}$$

where P_{Ch} and $[Ch]_o$ represent choline permeability and external choline concentration, respectively. The ratio P_{Ch}/P_{Na} was estimated to be 0.65 by fitting the measurements in Fig. 6 (broken curve) using Eq. (7). This value

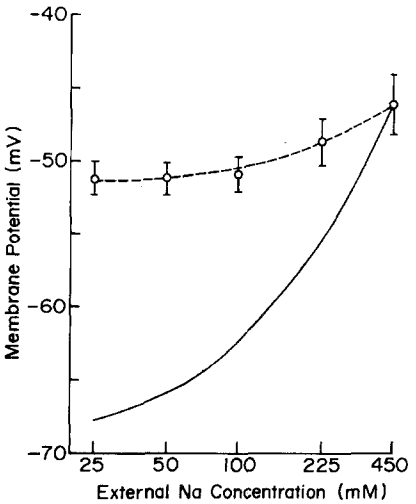


Fig. 6. Resting membrane potential as a function of external sodium concentration. Equimolar concentrations of choline were substituted for sodium in solutions with the sodium concentrations less than 450 mM. Circles represent the mean ±SEM (7 axons). Broken line was drawn by Eq. (7), and solid line by Eq. (5). The ratios P_{Na}/P_K, P_{Cl}/P_K and P_{Ch}/P_K in these equations are 0.039, 0.025 and 0.026, respectively

agrees reasonably well with the voltage clamp measurement which gave a value of 0.72.

Corrections of resting permeability ratios as measured by the membrane potential method. The permeability ratios P_X/P_{Na} obtained by the membrane potential method require corrections for choline and potassium permeabilities, because the choline permeability is not negligibly small with a ratio P_{Ch}/P_{Na} of 0.72 and the potassium permeability is affected by test cations to varying extents (Table 2). For the axons untreated with GTX I, corrections were made using the constant field Eq. (7) modified for various concentrations $[X]_o$. Firstly, the ratio P_{Na}/P_K was calculated for the same membrane potential as used to measure the ratio P_X/P_K by using Eq. (5) which applies with a ratio P_{Cl}/P_{Na} of 0.65 to normal sodium solutions without choline. Secondly, with all these values incorporated into Eq. (7), the ratios P_X/P_K were calculated from the values for E_m corresponding to two values for $[X]_o$. From the ratios P_X/P_K and P_{Na}/P_K thus obtained, the ratio P_X/P_{Na} was calculated. For cesium, guanidine and methylguanidine, the calculated ratios P_X/P_K were further corrected for the effects of these cations on P_K using the equation

$$\frac{P_X}{P_K}=\frac{\alpha}{[X]_o}\left\{\left([K]_i+\frac{P_{Na}}{\alpha P_K}[Na]_i\right)e^{E_m F/RT}-\left([K]_o+\frac{P_{Ch}}{P_{Na}}\frac{P_{Na}}{\alpha P_K}[Ch]_o\right)\right\} \quad (8)$$

in which α refers to the change in P_K caused by guanidine, methylguanidine and cesium in a value relative to the control (Table 2), and the ratios P_{Na}/P_K and P_{Ch}/P_{Na} were estimated from the measurements at two membrane potentials. The corrected values for P_X/P_{Na} are given in Table 3. It can be seen that these values are closer than the uncorrected values to those obtained by the voltage clamp method.

Corrections of the ratio P_X/P_{Na} in the GTX I-treated axons were made in a different manner, because the measurements for formamidine, guanidine and lithium which augmented the GTX I-induced depolarization had to be made at a membrane potential different from that for methylguanidine, methylamine and cesium which decreased the depolarization (Fig. 5*B*). The ratio P_{Na}/P_K was calculated from the equation

$$\frac{P_{Na}}{P_K}=\frac{[K]_i\, e^{E_m F/RT}-[K]_o}{[Na]_o+\frac{P_{Ch}}{P_{Na}}[Ch]_o-[Na]_i\, e^{E_m F/RT}} \tag{9}$$

where the ratio P_{Ch}/P_{Na} is estimated to be 0.43 by the voltage clamp method as described before. The next step was to calculate the ratio P_X/P_K from the measurements of the membrane potential in test cation solutions using Eq. (8). The values for $[X]_o$ and $[Ch]_o$ in Eq. (8) were obtained from the curve relating the membrane potential to the test cation concentration (Fig. 5*B*) at the same E_m value as used in Eq. (9). Thus the ratio P_X/P_{Na} was finally calculated from the values for P_{Na}/P_K and P_X/P_K. The corrections bring the ratios P_X/P_{Na} (Table 3) much closer to those obtained by the voltage clamp method (Table 1).

Ionic Permeabilities During Activity

Fig. 7 illustrates families of membrane currents associated with step depolarizations to various levels near the reversal potential for peak transient current in control Na sea water and in Cs solution outside. No transient current flowed at the membrane potentials of 40 mV and 15 mV in Na and Cs solutions, respectively. Similar measurements of the reversal potential were made with various test cations present outside.

The permeability ratios calculated from these data using Eq. (3) are given in Table 4. Before application of GTX I, lithium and sodium were almost equally permeant, formamidine and guanidine were moderately permeant (20% of P_{Na}), and cesium, methylguanidine and methylamine were only slightly permeant (less than 10% of P_{Na}). These permeability

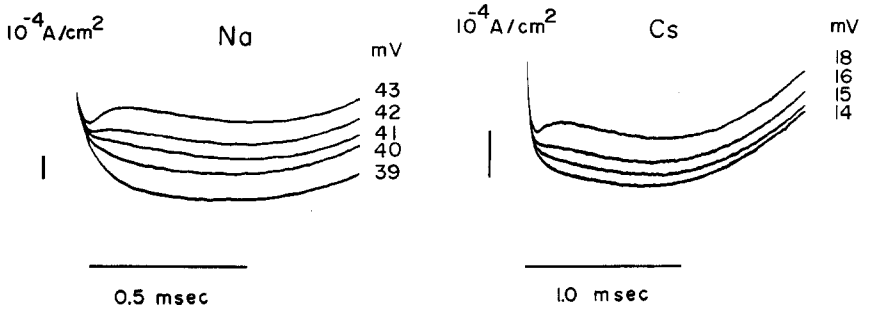


Fig. 7. Membrane currents associated with step depolarizations from the holding membrane potential of −80 mV to various levels indicated in normal Na sea water and a sea water in which Cs is substituted for Na

Table 4. Cation permeabilities (P_X) relative to sodium permeability (P_{Na}) during peak transient current before and during internal perfusion of 4×10^{-5} M grayanotoxin I (GTX I)

Cation	P_X/P_{Na}	
	Control	GTX I
Na	1.00	1.00
Li	1.12 ±0.025	1.03 ±0.078
Formamidine	0.20 ±0.012	0.18 ±0.019
Guanidine	0.20 ±0.014	0.19 ±0.022
Cs	0.085±0.029	0.067±0.011
Methylguanidine	0.061±0.012	0.063±0.010
Methylamine	0.036±0.003	0.040±0.004

Data are given in the mean ±SEM of 6 axons.

ratios were essentially unchanged after internal perfusion of GTX I at a concentration of 4×10^{-5} M.

Discussion

Two methods were used in the present study to estimate resting cation permeability ratios. One was based on the measurements of membrane currents under voltage clamp conditions (Narahashi & Seyama, 1974), and the other based on the calculations by the constant field equation from the data on the membrane potential (Hagiwara *et al.*, 1971). In the voltage clamp experiments, resting ionic permeabilities were calculated from the observed ionic currents by means of the constant field Eq. (4). This equation was used, since it represents one of the best first approxima-

tions despite several assumptions involved (Goldman, 1943; Hodgkin & Katz, 1949). It has been shown that the permeabilities measured by the voltage clamp method can be incorporated into the constant field equation to fit the observed values for the membrane potential (Fig. 2). The cation permeability ratios obtained by the two methods agree reasonably well if appropriate corrections are made for the calculations by the membrane potential method (Table 3). The voltage clamp method is more straightforward than the membrane potential method, because it does not require an inert substitute for changing test cation concentrations and is free from possible effects of the test cations on potassium and choline permeabilities (Table 2, Fig. 6). Another advantage of the voltage clamp method over the membrane potential method is that the permeability ratio P_X/P_{Na} can be measured at the same membrane potential enabling direct comparison of permeabilities to various test cations.

In the present study, cesium has been found to be less permeant in the resting condition than sodium before application of GTX I. This is at variance with previous observations by the membrane potential method in which cesium was found to be more permeant than sodium at resting conditions (Baker *et al.*, 1962; Adelman & Senft, 1968; Hagiwara *et al.*, 1972). It should be pointed out, however, that calculations of the ratio P_{Cs}/P_{Na} by the membrane potential method indeed give a high value of 4.79 without corrections for choline and potassium permeabilities (Table 3). Cesium does reduce potassium permeability (Table 2) and the substitute choline is permeant to the squid nerve membrane (Fig. 6). The corrections for these factors give a ratio P_{Cs}/P_{Na} of 0.92 which agrees well with the value of 0.87 obtained by the voltage clamp method.

Possible errors are involved in the measurements of resting permeability ratios by voltage clamp technique. First of all, the contributions of calcium current to the membrane current measured at -70 mV which is made equal to the equilibrium potentials for potassium and chloride would be negligibly small, since the ratio I_{Ca}/I_{Na} estimated from calcium and sodium fluxes is about 0.004 (Hodgkin & Keynes, 1955, 1957; Shanes & Berman, 1955). Although no measurements of the ratio I_{Ca}/I_{Na} have been made in the condition identical to that in the present study, the ratio can safely be assumed to be negligibly small in the perfused axons on the basis of very long survival times and small leakage conductance. The internally perfused axons usually maintained normal excitability for a longer period of time than the intact axons in terms of leakage conductance and sodium and potassium conductance increases associated with depolarizing stimulation.

Since potassium current contributes to a relatively large proportion of the resting membrane current at membrane potentials other than its equilibrium potential, a small error involved in the membrane potential measurement as a result of changes in junction potential could become significant in estimating the permeability ratio P_X/P_{Na} at the potassium equlibrium potential. Since the ratio P_X/P_{Na} is approximately equal to the ratio I'_X/I_{Na} in Eq. (6), the permeability ratios listed in Table 1 could actually represent the values (r) as given by the equation

$$r=\frac{I'_X+I_K}{I_{Na}+I_K} \tag{10}$$

if the membrane current measured contains a small amount of potassium current. Eq. (10) can be rewritten to give

$$\frac{I'_X}{I_{Na}}=r+\frac{I_K}{I_{Na}}(r-1) \tag{11}$$

where I_K/I_{Na} $(r-1)$ corresponds to the amount of the error involved in the permeability ratios given in Table 1. If the membrane potential were clamped at -75 mV instead of -70 mV, the average ratio I_K/I_{Na} would be 0.175 as calculated from the constant field Eq. (4) by incorporating the average P_{Na} and P_K values given in this paper. The errors involved in the ratios P_X/P_{Na} in Table 1 would then be 4, 3, 4, 5, 3 and 5% for lithium, cesium, formamidine, guanidine, methylguanidine and methylamine, respectively. The junction potential did not change more than 5 mV over a period of observations, so that the maximum amount of error caused by the contribution of potassium component to the membrane current would have been less than 5%. In the axons treated with GTX I, such errors should be much smaller, as sodium current is greatly increased.

The argument in the preceding paragraph also applies to the errors in the measurements of the ratios P_X/P_{Na} due to possible small differences in the activity coefficients between external and internal solutions. The errors in estimating the membrane potential due to such differences in the activity coefficients do not exceed 5 mV, so that the errors in the ratios P_X/P_{Na} would be less than 5%. Even when the maximum errors for the three major permeant ions sodium, potassium and chloride occur in the same direction and are added, the errors in the ratios P_X/P_{Na} would be less than 15%.

Possible asymmetric suppressions of potassium current by test cations could become a source of errors, but this remains to be seen in the future experiments.

It should be noted that GTX I does not drastically alter the resting cation permeability ratios (Table 1 and 3). At least three explanations are possible for this result. One possibility is that GTX I does not create a new ionic channel in the membrane but increases the number of resting ionic channels. The second possibility is that GTX I increases the mean opening time of individual sodium channels. The third possibility is that GTX I creates new ionic channels with field strength characteristics and spatial array very similar to those of the preexisting ionic channel (*cf.* Eisenman, Szabo, Ciani, McLaughlin & Krasne, 1973). The present study does not permit distinguishing these three possibilities.

The cation permeability ratios at rest vary in a relatively narrow range, from a ratio P_X/P_{Na} of 0.78 for the least permeant methylamine to 1.49 for the most permeant guanidine (Table 1). Even choline is permeant to about the same extent as methylamine with a ratio of P_{Ch}/P_{Na} of 0.73. However, the low specificity of the squid nerve membrane for passage of various cations does not mean that these cations can cross the membrane at the rates determined by their mobilities in solutions, because the permeability sequence described in this paper is entirely different from the mobility sequence in solutions (Scudder, 1914; Robinson & Stokes, 1965).

It is worth emphasizing that the permeability ratios and sequences in the resting nerve membrane before and during application of GTX I are strikingly different from those during activity. Table 5 summarizes perme-

Table 5. Permeability ratios during peak current and at rest in frog nodes of Ranvier and squid axons

	Peak current			At rest	
	Frog[a]	Squid[b]	Squid[c]	Squid[d]	Squid[c]
Na	1.0	1.0	1.0	1.0	1.0
Li	0.93	1.1	1.12	~1.0	0.83
Formamidine	0.14	–	0.20	–	1.34
Guanidine	0.13	–	0.20	–	1.49
Cs	<0.013	0.017	0.085	3.6	0.87
Methylguanidine	<0.01	–	0.061	–	0.86
Methylamine	<0.007	–	0.036	–	0.78
Choline	<0.007	–	–	–	0.73

[a] Hille (1971, 1972)
[b] Chandler & Meves (1965)
[c] This paper
[d] Calculated from Baker *et al.* (1964) and Hagiwara *et al.* (1972)

ability ratios during peak transient activity and at resting conditions in frog nodes of Ranvier and squid giant axons. Both the frog nodes and squid axons exhibit very similar permeability ratios during activity with a very high selectivity for certain cations including sodium and lithium. These membranes are poorly permeable to some other cations during activity including cesium, methylguanidine, methylamine and choline. However, the latter group of relatively impermeant cations is indeed permeant to the resting squid axon membrane with a ratio P_X/P_{Na} of 0.73–0.87. The cation permeability ratios in the squid axon membrane at rest and during activity are maintained after exposure to GTX I despite the drastic increase in the absolute value for resting cation permeability (Table 1). In terms of Eisenman's selectivity isotherms (Eisenman, 1967; Eisenman *et al.*, 1973), the nerve membrane exhibits the permeability sequence *X* with a high field strength during peak transient current, whereas it exhibits the permeability sequence IV or V with an intermediate field strength at resting conditions both before and during exposure to GTX I. These observations are compatible with the notion that the "resting" sodium channel is operationally different from the sodium channel that undergoes a conductance increase upon electrical stimulation (Narahashi *et al.*, 1971).

An alternative possibility is that the selectivity for cation permeabilities of sodium channels is affected by the membrane potential through alteration of the field strength in the membrane. According to this hypothesis, the cation selectivity of sodium channels is low at -70 mV, and is expected to increase with depolarization attaining a high value near the reversal potential for peak transient current. In Eisenman's selectivity isotherms (Eisenman, 1967; Eisenman *et al.*, 1973), the ratio P_{Na}/P_K drastically increases from a value of about 1/10 at rest (sequence IV or V) to a value of about 10 during activity (sequence *X*). However, the ratio P_{Na}/P_K during peak transient current of squid axons was indeed independent of the reversal potential (Cahalan and Begenisich, 1975). Thus this observation, although limited to a narrow range of the membrane potential, does not lend support to the alternative hypothesis. It would be necessary to extend such measurements over a wider range of the membrane potential to make a comparison of permeability ratios at rest and during activity. It would also be necessary to study the resting cation permeability selectivity at membrane potentials more positive than -70 mV where the present measurements were made.

Two other observations are also in keeping with the notion of separate sodium channels. Although tetrodotoxin antagonizes the resting sodium conductance increase caused by GTX I, the concentration of tetrodotoxin

required for this action is much higher with the apparent dissociation constant of 4×10^{-8} M (Narahashi & Seyama, 1974) than that required for blockage of peak transient current with the apparent dissociation constant of 3×10^{-9} M (Cuervo & Adelman, 1970). The difference is difficult to account for on the basis of single sodium channel. The steady-state sodium inactivation curve for the peak transient current is shifted by GTX I in the direction of hyperpolarization thereby increasing the proportion of the peak transient sodium mechanism that is in an inactivated state at resting conditions (Seyama & Narahashi, 1976). This occurs in the face of an increase in resting sodium conductance which cannot be inactivated. This observation is also in favor of the separate sodium channel concept.

The resting potassium permeability is increased only by a factor of 1.24 during application of GTX I. This amount of permeability increase is negligible compared with the permeability increase to sodium and other test cations which is on the order of 10-fold. Such differential action of GTX I lends support to the notion that the resting sodium channel is a different entity from the resting potassium channel or the potassium channel that undergoes a permeability increase upon depolarization. The observation that α-dihydrograyanotoxin II slightly inhibits the voltage dependent potassium conductance increase during depolarization (Seyama & Narahashi, 1973) also supports this notion.

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