Effects of Batrachotoxin on Membrane Potential and Conductance of Squid Giant Axons

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ABSTRACT The effects of batrachotoxin (BTX) on the membrane potential and conductances of squid giant axons have been studied by means of intracellular microelectrode recording, internal perfusion, and voltage clamp techniques. BTX (550–1100 nM) caused a marked and irreversible depolarization of the nerve membrane, the membrane potential being eventually reversed in polarity by as much as 15 mv. The depolarization progressed more rapidly with internal application than with external application of BTX to the axon. External application of tetrodotoxin (1000 nM) completely restored the BTX depolarization. Removal or drastic reduction of external sodium caused a hyperpolarization of the BTX-poisoned membrane. However, no change in the resting membrane potential occurred when BTX was applied in the absence of sodium ions in both external and internal phases. These observations demonstrate that BTX specifically increases the resting sodium permeability of the squid axon membrane. Despite such an increase in resting sodium permeability, the BTX-poisoned membrane was still capable of undergoing a large sodium permeability increase of normal magnitude upon depolarizing stimulation provided that the membrane potential was brought back to the original or higher level. The possibility that a single sodium channel is operative for both the resting sodium permeability and the sodium permeability increase upon stimulation is discussed.

INTRODUCTION

Batrachotoxin (BTX) is one of the toxic principles contained in the skin secretion of the Colombian arrow poison frog, Phyllobates aurotaenia (Märki and Witkop, 1963; Daly, Witkop, Bommer, and Biemann, 1965; Tokuyama, Daly, Witkop, Karle, and Karle, 1968; Tokuyama, Daly, and Witkop, 1969). Recent studies with purified BTX samples demonstrated that the poison
caused a depolarization of mammalian skeletal muscle membranes, an increase and subsequent block of spontaneous and stimulus-evoked transmitter release, and a simultaneous increase in muscle tension (Albuquerque, Warnick, and Sansone, 1971; Warnick, Albuquerque, and Sansone, 1971). Heart Purkinje fibers were found to be more sensitive to BTX than mammalian skeletal muscle fibers (Hogan and Albuquerque, 1971). Batrachotoxin caused an initial alteration in the shape of the cardiac action potential followed by a depolarization of the membrane, and on many occasions the resting membrane potential was reversed in polarity, the inside becoming positive with respect to the outside. All these effects of BTX on cardiac and skeletal muscles were completely antagonized by tetrodotoxin (TTX); it was suggested that BTX acted on presynaptic nerve membrane, postsynaptic muscle membrane, and heart Purkinje fiber membrane by specifically increasing sodium permeability.

The purpose of the present study is to elucidate the detailed ionic mechanism of action of BTX on the membrane of squid giant axons by means of internal perfusion and voltage clamp techniques. Experiments on the resting membrane potential carried out with intact and internally perfused squid axons have led us to the conclusion that the depolarization of the nerve membrane produced by application of BTX is due primarily to an increase in resting sodium permeability. Voltage clamp experiments have demonstrated that the axon membrane treated with BTX, in the face of the increased resting sodium permeability, is still capable of undergoing a further sodium permeability increase upon depolarization, provided that the membrane potential is brought back to the original level by application of a hyperpolarizing current.

Preliminary accounts of this work have already been published (Narahashi, Albuquerque, and Deguchi, 1970; Narahashi, Deguchi, and Albuquerque, 1971).

METHODS

Material Giant axons from the squid, Loligo pealei, available at the Marine Biological Laboratory, Woods Hole, Massachusetts, were used in all experiments.

Intact Axons Isolated axon was cleaned by removing thin nerve fibers and connective tissues, mounted in a nerve chamber, and continuously perfused externally with either control or test solutions. The resting membrane potential was recorded by means of conventional intracellular microelectrode techniques. The microelectrodes were filled with 3 M KCl and selected for a resistance of 5-10 MΩ. Electrical stimulation was applied via a pair of Ag-AgCl wire electrodes located near one end of the axon.

Internally Perfused Axons The method of internal perfusion was essentially the same as that described previously (Narahashi and Anderson, 1967). A platinum wire having a diameter of 50 μm plated with platinum black was used instead of an Ag-AgCl wire for current delivery in perfused axons. Both external and internal media were perfused continuously throughout the experiment.
Voltage Clamp. Voltage clamp experiments with intact squid axons were performed using a sucrose-gap chamber by the method similar to that described previously (Moore, Narahashi, and Ulbricht, 1964).

Temperature. Since the action of BTX has a high Q10 value (Warnick et al., 1971), most of the present experiments on the resting membrane potential were performed at room temperature (23°C). However, some experiments were done at 15°C; in this case the time course of membrane depolarization was somewhat slower but eventually attained the same level. Voltage clamp experiments were carried out at 12°C.

Solutions and Drugs. The compositions of physiological saline solutions used are given in Table I. Batrachotoxin (C₉H₄N₂O₆) is batrachotoxin A 20α-2,4-dimethylpyrrole-3-carboxylate; batrachotoxin A is 3α, 9α-epoxy-14β, 18β-(epoxyethano-N-

Table I

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* In the form of KH₂PO₄.

methylimino)-5β-pregna-7,16-diene-3β, 11α, 20α-triol (Tokuyama et al., 1969). The molecular weight of BTX is 538 and the pKₐ is 7.45. Tetrodotoxin was purchased from Sankyo Co., Ltd., Japan, via Calbiochem, Los Angeles, Calif. Tris [tris(hydroxymethyl) aminomethane] was purchased from Sigma Chemical Co., St. Louis, Mo. All stock solutions were kept refrigerated until immediately before use.

RESULTS

Effects of Batrachotoxin on Resting Membrane Potential

External application of BTX to the squid giant axon in a concentration of 1100 nM caused an irreversible depolarization of the membrane. The membrane depolarization progressed slowly but steadily, and after about 1 hr of exposure to the toxin the membrane potential was in many cases reversed in polarity by several millivolts. Although internal perfusion of the axon with a
solution containing BTX in a concentration of 550 nM produced a membrane depolarization similar to that when the poison was applied externally, the depolarization progressed more rapidly in the former than in the latter.

Fig. 1 illustrates a typical experiment in which BTX (550 nM) was added to the standard internal solution (SIS) containing 50 mM Na. The results of this experiment can be summarized as follows: (a) Before application of BTX,
the membrane was even hyperpolarized by 10 mV beyond the level achieved in 1 mM Na before application of BTX. (d) Complete recovery of the resting membrane potential of the BTX-treated axon was brought about by 1000 nM TTX-ASW.

In other preparations, external application of TTX (1000 nM) or 1 mM Na prevented the nerve membrane from being depolarized by the subsequent application of BTX either externally or internally (Fig. 2). However, the

### Table II

<table>
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<th>Calculation</th>
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<tr>
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Calculations were made by equation (3) (ASW, artificial seawater; TTX, tetrodotoxin; BTX, batrachotoxin). Observed values are given in the mean ± SE of the mean with the number of measurements in parentheses.

* BTX (1100 nM) and TTX (1000 nM) were applied externally.
† Maximum depolarization.
§ BTX (550 nM) was applied internally and TTX (1000 nM) applied externally. Internal perfusate contains 50 mM Na.

The mean values of the resting membrane potential in intact and internally perfused axons under various experimental conditions are given in Table II.
A slight hyperpolarization of the nerve membrane caused by external application of TTX or 1 mM Na confirms the previous observation by Freeman (1969) and suggests that TTX inhibits the resting sodium permeability (see Discussion for further details). Since the equilibrium potential for sodium in 1 mM Na is calculated to be $-100$ mv, the aforementioned results with BTX are compatible with the notion that changes in the membrane potential are due primarily to an increase in resting sodium permeability of the nerve membrane. If this hypothesis were correct, the membrane potential would not be affected at all when BTX was applied to the axon in the absence of sodium in both external and internal phases. This has proved to be the case (see Fig. 2 of Narahashi et al., 1971). Before and after application of 550 nM BTX internally, the mean resting membrane potentials were $-62.7 \pm 3.27$ mv (four measurements) and $-63.3 \pm 2.19$ mv (eight measurements), respectively. In one experiment, 1000 nM TTX was applied internally to the BTX-perfused axon in the absence of sodium in both phases; there was no effect on the resting membrane potential.

These experiments with BTX in the absence of sodium in both phases demonstrate that an increase in the resting sodium permeability is the major action of BTX responsible for a large depolarization which occurs when the normal concentration of sodium is present outside.

**Effects of Batrachotoxin on Action Potential**

During the course of membrane depolarization produced by BTX applied either outside or inside the axon, the action potential gradually decreased in amplitude and eventually was blocked. However, when the membrane potential was brought back to the original level by application of an inward current across the membrane, action potentials with normal amplitude could be elicited. Although the rising phase and the initial part of the falling phase of the action potential were almost unaffected by BTX, the undershoot that followed the spike phase in control preparations disappeared in poisoned axons, and the spike was followed by a prolonged repolarizing phase or negative afterpotential. The large negative afterpotential lasted several seconds, and in some cases as long as 1 min (Fig. 3). Spike activity occurred spontaneously in some axons when the membrane potential of the BTX-treated preparation was maintained at a high level by application of hyperpolarizing current. In these experiments a cycle of spontaneous depolarization and repolarization was repeated without being preceded by a spike (Fig. 3).

It is noteworthy that the axon membrane treated with BTX remained depolarized following repetitive stimuli under sucrose-gap conditions. In the control preparation before treatment with BTX, a burst of repetitive stimuli at a frequency of 5 cycles per sec did not produce any persistent depolarization following cessation of the electrical stimuli. During the initial stage of BTX
poisoning at a time when the axon membrane began to depolarize, a burst of repetitive stimuli caused the negative afterpotentials to be built up, and upon cessation of the stimuli the membrane remained depolarized. The axon membrane was then gradually repolarized toward the resting potential level. After a longer period of exposure to BTX, the effect of repetitive stimuli became more pronounced, and the negative afterpotentials were built up much more rapidly. Upon termination of the repetitive stimuli, the nerve membrane remained at a greatly depolarized level, and recovered only to a very small extent. It appears that the membrane conductance rapidly increases during repetitive stimuli, so that the hyperpolarizing current created by the sucrose gap becomes much less effective in maintaining the membrane potential at a high level.

**Effects of Batrachotoxin on Membrane Currents and Conductances**

**IN NORMAL EXTERNAL SODIUM CONCENTRATION** As might be expected from the observations of the action potential described above, the BTX-poisoned nerve membrane was still capable of producing the peak transient sodium current under voltage clamp conditions provided that the membrane potential was brought back to high levels. Examples of families of membrane currents associated with step depolarizations of various magnitudes before and after application of BTX are illustrated in Fig. 4. The amplitude of the peak sodium current was not significantly affected by the treatment with BTX, whereas the steady-state potassium current was suppressed to 70% control.

The tail current that followed the cessation of depolarizing pulse was decreased in amplitude by application of BTX to 55% control in the experiment illustrated in Fig. 4. The membrane was held, between depolarizing pulses, at −80 mv which was the average value for the equilibrium potential for the steady-state current as will be described later. The late membrane current in the control experiment of Fig. 4 did not decrease during depolarization, yet the inward tail current was produced. This suggests that the equilib-
Equilibrium potential for the steady-state current in this axon was less negative than -80 mv, and that the tail current was carried by potassium ions. Therefore the decrease in tail current caused by BTX can be interpreted as being associated with the decrease in the steady-state current.

The peak amplitude of the sodium current and the steady-state amplitude of the potassium current are plotted as a function of the membrane potential in Fig. 5. The amplitude of the peak sodium current was not significantly affected by BTX, whereas the steady-state potassium current was suppressed to 70% control at the membrane potential of 100 mv. Another change worthy of note is the shift that was observed in the current-voltage curves along the potential axis in the direction of depolarization by exposure to BTX. The curve for the peak sodium current was shifted by approximately 15 mv at inside negative membrane potentials, but the shift was only 4 mv at more depolarized membrane potential levels. The steady-state potassium curve was shifted 15–30 mv at all membrane potential levels examined.

The effects of BTX on the characteristics of membrane conductances are more clearly seen when the membrane conductances are plotted on a logarithmic scale as a function of the membrane potential (Fig. 6). The conductances were calculated from the equations:

\[ g_p = \frac{I_p}{(E - E_p)} \]  
\[ g_{ss} = \frac{I_{ss}}{(E - E_{ss})} \]
FIGURE 5. Current-voltage relations for the peak transient sodium current ($I_p$) and for the steady-state potassium current ($I_{ss}$) in an intact squid giant axon before and during external application of 550 nM batrachotoxin (BTX). ASW, artificial seawater. The currents are not corrected for leakage current ($I_l$) whose steady-state value is plotted separately.

FIGURE 6. Peak transient sodium conductance ($g_p$) and steady-state potassium conductance ($g_{ss}$) plotted on a logarithmic scale as a function of the membrane potential before and during external application of 550 nM batrachotoxin (BTX). Intact squid giant axon. ASW, artificial seawater.
where \( g, I, \) and \( E \) with subscript refer to the membrane conductance, membrane current, and equilibrium potential, respectively, the subscripts \( p \) and \( ss \) refer to peak and steady state, respectively, and \( E \) without subscript represents the membrane potential. The value of \( E_p \) was measured at the membrane potential where \( I_p \) intersected the potential axis at zero current.

The value of \( E_{ss} \) was not measured in each experiment, but a few separate measurements gave an average value of \(-80 \text{ mV}\). The measurements of \( E_{ss} \) were made in the following way: The membrane was first depolarized from the holding membrane potential of \(-80 \text{ mV}\) to \(0 \text{ mV}\), and repolarized at \(2\) msec after the onset of the pulse to various levels ranging from \(-100 \text{ mV}\) to \(-60 \text{ mV}\). The membrane potential at which the steady-state potassium current was not followed by a tail current was taken as a measure of \( E_{ss} \).

A vertical shift of the conductance curve in Fig. 6 indicates an effect of BTX on the magnitude of the membrane conductance, and a horizontal shift after normalization indicates a shift of the conductance curve along the potential axis. The latter shift was measured at the level where the conductance was half-maximum.

The following mean values (± standard error of mean) of \( g_p \) and \( g_{ss} \), in a value relative to control, were obtained from seven axons perfused externally with \(550 \text{ nM BTX-ASW}: g_p \) \(1.04 \pm 0.10\); \( g_{ss} \) \(0.78 \pm 0.13\). The average shift of the conductance curve along the potential axis in those axons was \( g_p \) \(9.5 \pm 1.8 \text{ mV}\) in the direction of depolarization; \( g_{ss} \) \(8.1 \pm 2.5 \text{ mV}\) in the direction of depolarization. The shift of \( E_p \) caused by BTX was variable ranging between \(12 \text{ mV}\) in the direction of depolarization and \(22 \text{ mV}\) in the direction of hyperpolarization with the mean value of \(2.1 \pm 3.4 \text{ mV}\) in the direction of hyperpolarization.

In \(1 \text{ mM external sodium solutions}\) Families of membrane currents associated with step depolarizations from the holding membrane potential of \(-80 \text{ mV}\) before and after external application of \(550 \text{ nM BTX}\) are illustrated in Fig. 7. BTX had little or no effect on the outward peak sodium current and on the outward steady-state potassium current.

Current-voltage relationships for the peak sodium current and for the steady-state potassium current are shown in Fig. 8. In Fig. 9 each component of the conductances is plotted on a logarithmic scale as a function of the membrane potential. The values of \( g_p \) and \( g_{ss} \) from four axons, in a value relative to control, were estimated to be \(0.94 \pm 0.11\) and \(0.84 \pm 0.13\), respectively (mean ± standard error of mean). These effects of BTX are almost the same as those observed in the presence of normal external sodium concentration.

The shift of \( g_p \) and \( g_{ss} \) curves along the potential axis by application of BTX was very small. The mean values (± standard error of mean) were estimated to be \(0.5 \pm 1.4 \text{ mV}\) for \( g_p \) and \(1.2 \pm 1.8 \text{ mV}\) for \( g_{ss} \), both being in the direc-
tion of depolarization. This is in contrast with the observation in the presence of normal sodium concentration outside where a large shift was brought about in the direction of depolarization.

**Leakage Current**  If the resting sodium permeability is increased by application of BTX, then the leakage current measured under voltage clamp conditions would be expected to increase. However, since the resting sodium permeability in normal squid axons is only a few per cent of the total ionic permeability, a small increase in sodium permeability might not be readily detected by measurements of leakage current.
In three voltage clamp experiments, changes of the leakage current associated with a step hyperpolarization of 20-80 mv were measured during the initial period of BTX action. In all three cases, the leakage conductance was found to increase after exposure to BTX: from 3.1 to 3.9 mmho/cm², from 3.5 to 7.6 mmho/cm², and from 2.9 to 4.4 mmho/cm². However, with the advance of time after exposure to BTX, the leakage current began to decrease and eventually an outward steady current was observed upon step hyperpolarization of 20-80 mv from the holding membrane potential of -80 mv. Because of such peculiar behavior of leakage current in BTX-treated axons, no correction for leakage current was made in presenting the peak sodium and steady-state potassium currents. The mechanism underlying the outward leakage current in BTX-treated axons remains to be explored.

**Figure 9.** Peak transient sodium conductance ($g_p$) and steady-state potassium conductance ($g_s$) plotted on a logarithmic scale as a function of the membrane potential before and during external application of 550 nM batrachotoxin (BTX). Intact squid giant axon bathed in solution that has 1 mM Na.

**Discussion**

The present results demonstrate that exposure of the squid giant axon to BTX causes a membrane depolarization which is dependent upon the presence of sodium ions in the external solution. The membrane depolarization produced by BTX progresses irreversibly to a positive value of 15 mv or more. This depolarization is quickly and completely antagonized by either external application of TTX or removal or drastic reduction of external sodium concentration. Furthermore, BTX does not exert any effect on the resting membrane potential provided that sodium ions are lacking in both external and internal phases of the axon. These observations clearly demonstrate that the depolarization of the squid axon membrane caused by BTX is due to a specific
increase in resting sodium permeability. The observations with the pre- and postsynaptic membranes of skeletal muscle (Warnick et al., 1971; Albuquerque et al., 1971) and with heart Purkinje fibers (Hogan and Albuquerque, 1971) lend support to the same mechanism of action of BTX on these fibers.

The possibility of a decrease in resting potassium permeability ($P_K$) can be excluded for the following two reasons: (a) Since TTX does not affect $P_K$, it would have failed to restore the BTX depolarization if the latter were primarily due to a decrease in $P_K$; (b) the values of the equilibrium potential for sodium ($E_{Na}$), potassium ($E_K$), leakage ($E_l$), and chloride ($E_{Cl}$) in the squid axon bathed in 1 mM Na solution are calculated to be $-100 \text{ mV}$, $-92 \text{ mV}$, $-50 \text{ mV}$ (Hodgkin and Huxley, 1952), and $-39 \text{ mV}$ (Keynes, 1962), respectively. Since the leakage permeability ($P_l$) or chloride permeability ($P_{Cl}$) is much greater than the sodium permeability ($P_{Na}$) under normal resting conditions, the nerve membrane would have been depolarized in BTX-1 mM Na if the decrease of $P_K$ were the major effect of BTX. However, the nerve membrane was in fact hyperpolarized by application of BTX-1 mM Na.

The possibility of an increase in $P_l$ or $P_{Cl}$ can also be excluded for two reasons: (a) If the depolarization caused by BTX were primarily due to an increase in $P_l$ or $P_{Cl}$, the membrane potential would have been maintained near $E_l (-50 \text{ mV})$ or $E_{Cl} (-39 \text{ mV})$. However, the nerve membrane was in fact depolarized far beyond those levels; (b) since TTX had no effect on the leakage current (Moore, Narahashi, Poston, and Arispe, 1970), the depolarization induced by BTX would not have been restored by application of TTX if the increase in $P_l$ were the major action of BTX.

In order to obtain a more quantitative basis for the above-mentioned explanation for the BTX action, an attempt was made to calculate the relative permeability changes produced by application of BTX. The following Goldman-Hodgkin-Katz constant-field equation (Hodgkin and Katz, 1949) was used for intact axons:

$$E = \frac{RT}{F} \ln \frac{P_K(a_K)_o + P_{Na}(a_{Na})_o + P_{Cl}(a_{Cl})_i}{P_K(a_K)_i + P_{Na}(a_{Na})_i + P_{Cl}(a_{Cl})_o}$$          (3)

where $E$, $R$, $T$, and $F$ represent the membrane potential, gas constant, absolute temperature, and Faraday constant, respectively, $(a_K)$, $(a_{Na})$, and $(a_{Cl})$ represent the activities of K, Na, and Cl, respectively, with subscripts o and i referring to outside and inside phases, respectively. Thus $(a_K)_o$, $(a_{Na})_o$, and $(a_{Cl})_o$ in ASW were 0.0068, 0.305, and 0.388, respectively, assuming the activity coefficient of 0.68 (Baker, Hodgkin, and Meves, 1964). The values of $(a_K)_i$ and $(a_{Na})_i$ were estimated as 0.203 and 0.0374, respectively (Hinke, 1961). The value of $(a_{Cl})_i$ was calculated as 0.0798 from the internal chloride concentration of 114 mM and the activity coefficient of 0.7 (Keynes, 1963).
For calculations of the membrane potential by using equation (3), the ratio $P_K:P_{Na}:P_{Cl}$ is assumed as follows: When the intact axon is bathed in normal ASW, $P_K:P_{Na}:P_{Cl}$ is equal to 1:0.035:0.02. Tetrodotoxin suppresses $P_{Na}$ selectively making $P_K:P_{Na}:P_{Cl}$ equal to 1:0.025:0.02 (Freeman, 1969; Baker, Blaustein, Keynes, Manil, Shaw, and Steinhardt, 1969). Batrachotoxin increases $P_{Na}$, and the depolarization due to this increase of $P_{Na}$ in turn causes an increase in $P_K$. Since the effect of BTX proceeds rather slowly, calculations are made at two stages of BTX poisoning. Thus at an initial stage, $P_K:P_{Na}:P_{Cl}$ is equal to 5:1:0.02, and at a later stage $P_K:P_{Na}:P_{Cl}$ is equal to 5:3.5:0.02. Addition of TTX to BTX decreases $P_{Na}$ to the value attainable when TTX alone is applied. Hence, $P_K:P_{Na}:P_{Cl}$ is equal to 1:0.025:0.02.

When the intact axon is bathed in 1 mM Na, the membrane resistance increases 20% (Freeman, 1969). When this change is taken into account, $P_K:P_{Na}:P_{Cl}$ equal to 0.6:0.035:0.1 is chosen to fit the observation. The effects on permeabilities of TTX, BTX, or TTX plus BTX in 1 mM Na are assumed to be the same as those in ASW.

For the internally perfused axons bathed in ASW, $P_K:P_{Na}:P_{Cl}$ equal to 1:0.09:0.02 is used to fit the observed value. Then the ratio is changed in various test solutions in the same manner as in the intact axon.

The results of calculations of the membrane potential are given in Table II together with the average observed values. The calculations fit the observations reasonably well for intact axons except for the value in 1 mM Na added with TTX and BTX where the calculated membrane potential is about 8 mv less negative than the observed one. For internally perfused axons, the calculations fit the observations when the external solution contained sodium ions at normal concentration. However, when 1 mM Na solution was used for the bathing medium, the calculated values were more inside negative than the observed values by 40-44 mv. This is presumably due to the fact that $P_{Cl}$ is used in lieu of $P_t$, because $E_K$ and $E_{Na}$ are inside negative and $E_{Cl}$ is negative infinity under these conditions. In the absence of data concerning the kinds of ions involved in the leakage conductance in perfused axons bathed in 1 mM Na solution, calculations of the membrane potential under these conditions await further study.

Strong antagonistic action of TTX on the depolarization caused by application of BTX is worthy of note. Tetrodotoxin by itself slightly hyperpolarizes the squid axon membrane in the presence of normal sodium concentration in the external phase, but lacks this action if external sodium concentration is decreased to 1 mM by substitution with Tris (Freeman, 1969; present study). Tetrodotoxin was also found to partially decrease the sodium influx from 27.6 to 11.2 pmole/cm² sec (Baker et al., 1969). Therefore, it is assumed that TTX decreases the resting sodium permeability. This action of TTX seems to be exerted in the presence of BTX which increases the resting sodium permeabil-
ity. It should also be noted that TTX blocks the increase in sodium permeability caused by depolarizing stimulation both in the presence and in the absence of BTX.

The amplitude of the action potential and the peak sodium current remains unaffected by treatment with BTX provided that the membrane potential is brought back to the original control level by anodal hyperpolarization. This observation and that of Hogan and Albuquerque (1971) raise the possibility that the resting sodium permeability is operationally different from the sodium permeability that undergoes a marked increase upon stimulation. However, the following observations are perhaps more in favor of the notion that there is a single sodium channel:

1. TTX decreases the resting $P_{Na}$ and inhibits the increase in $P_{Na}$ upon stimulation.

2. During the initial period of BTX poisoning, the membrane is kept depolarized upon cessation of repetitive stimuli. This is not observed in normal control preparations. This sustained afterdepolarization under sucrose-gap conditions is presumably due to a sustained increase in membrane conductance following repetitive stimuli. The hyperpolarizing current created by the sucrose gap would then become much less effective in maintaining the membrane potential at a high level, thereby producing an afterdepolarization. If the increase and prolongation of the negative afterpotential in the BTX-poisoned axon were due to a sustained increase in $P_{Na}$, the maintained depolarization after repetitive stimuli would be more compatible with the single sodium channel concept.

3. The resting $P_{Na}$ is expected to increase some 100-fold to cause the great depolarization observed in BTX-treated axons. The ratio $P_{K}:P_{Na}:P_{Cl}$ in intact squid axons is assumed as $1:0.035:0.02$ to fit the observed value for the resting membrane potential. If one takes 3 mmho/cm$^2$ as the conductance of the resting nerve membrane, the resting $g_{Na}$ is calculated to be 0.1 mmho/cm$^2$. The resting $g_{Na}$ will be increased to 10 mmho/cm$^2$ by a factor of 100 by application of BTX. Under voltage clamp conditions, the membrane conductance during the peak transient current of normal and BTX-poisoned axons could increase to 100 mmho/cm$^2$ upon depolarization. Since the peak current is carried mostly by sodium ions, $g_{Na}$ in BTX-poisoned axons is increased from 10 mmho/cm$^2$ to 100 mmho/cm$^2$ during depolarizing stimulation. Thus the net increase in $g_{Na}$ caused by depolarizing stimulation is 90 mmho/cm$^2$ in BTX-poisoned axons as against 99.9 mmho/cm$^2$ in normal axons. The value of $g_{Na}$ in BTX, in a value relative to control, is calculated to be 0.90 as against the observed value of 1.04 ± 0.10; these two values are within the experimental error.

These observations can be accounted for without assuming separate sodium channels in the nerve membrane at rest and during activity. However, no
experimental evidence is presently available to completely exclude one of the two possibilities.

The mechanism involved in the augmentation and prolongation of the negative afterpotential in BTX-poisoned axons remains to be studied. Although no detailed experimental analyses were performed in the present study, one voltage clamp experiment with depolarizing pulses of 200 msec duration showed that a residual sodium current was flowing during the maintained depolarization. External application of TTX abolished the residual sodium current.

We are indebted to Drs. J. Daly and B. Witkop for the supply of batrachotoxin samples. Thanks are also due to Mr. Robert C. de Groof for his analyses of voltage clamp data, Mr. Edward M. Harris for his maintenance of electronic equipment, Mrs. Donna Crutchfield, Mrs. Delilah Munday, and Miss Mabel A. Zelle for their secretarial assistance.

This study was supported by grants from the National Institutes of Health (NS03437 and NS08233), and the experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts.

Received for publication 2 February 1971.

REFERENCES


NARAHASHI, T., E. X. ALBUQUERQUE, and T. DEGUCHI. 1970. Effects of batrachotoxin on ionic


