

## Effect of batrachotoxin on the electroplax of electric eel: Evidence for voltage-dependent interaction with sodium channels\*

(membrane depolarization/action potentials)

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**ABSTRACT** Batrachotoxin under certain conditions has a strong depolarizing effect on the innervated membrane of the monocellular electroplax preparation from the electric eel, *Electrophorus electricus*. No effect is observed when the toxin (50–200 nM) is applied to the resting membrane for periods up to 1 hr. However, if the membrane is exposed to batrachotoxin and the cell is subjected to stimulation at a stimulus voltage slightly above the threshold for action potential firing, a progressive prolongation of the action potential and concomitant progressive depolarization of the innervated membrane is observed. When the membrane is depolarized by 15–20 mV, a further abrupt all-or-none depolarization occurs, and the potential attains a steady-state value between 0 and –10 mV. Brief stimulation of a cell in the presence of batrachotoxin is sufficient to define a batrachotoxin-treated cell, even though negligible depolarization occurs. If depolarizing agents such as carbamoylcholine or potassium chloride are introduced to such a cell in concentrations that normally produce a 20–30 mV depolarization, the abrupt all-or-none depolarization immediately occurs. All-or-none depolarizations arising from either electrical stimulation or depolarizing agents are unaffected by *d*-tubocurarine but are completely reversed by tetrodotoxin. Batrachotoxin thus appears to activate only the action potential sodium channels. In the batrachotoxin-treated membrane, these channels can attain stable steady states in either a closed configuration at the normal resting potential or in an open configuration after complete depolarization. A striking hysteresis cycle thus can be generated, which is strongly indicative of a voltage-dependent interaction of the toxin with the action potential sodium channels.

The molecular components that control ion conductances across excitable membranes of nerve and muscle cells have become increasingly accessible to biochemical studies. Of primary importance in this development is the introduction of a variety of naturally occurring toxic compounds, many of which appear to have high affinity for a specific component in excitable membranes. Among these toxic molecular probes is batrachotoxin (BTX), a novel steroidal alkaloid obtained from the skin of the Colombian dendrobatid frog *Phyllobates aurotaenia*. According to current views, BTX is an extremely potent, selective, and virtually irreversible activator of sodium channels<sup>||</sup> in excitable membranes (2). BTX appears to act at the same site on the action potential sodium channels as the less potent plant alkaloid veratridine. Tetrodotoxin (TTX), a toxin occurring in some fish and amphibians, acts noncompetitively at a separate site on these channels to inhibit the action of either activator (3).

Abbreviations: BTX, batrachotoxin; TTX, tetrodotoxin; NR, eel saline solution (normal Ringer's); St, stimulation.

\* Part of this work has appeared in a preliminary report (1).

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<sup>||</sup> The term "sodium channels" in this paper refers exclusively to the specific sodium channels that undergo transient conductance increases during action potential firing in excitable membranes.

The innervated face of the isolated electroplax preparation consists of two classes of excitable membranes (4); see ref. 5. Conducting membrane, analogous to nerve axonal membrane, may be induced to fire a propagated action potential by an imposed outward current flow (cathodal stimulation). Synaptic membrane, which is localized at thousands of residual nerve terminals scattered across the innervated face, may be depolarized following either anodal stimulation of the nerve terminals or direct application of cholinergic agonists. Previous work in our laboratory suggests that veratridine can activate both sodium channels and synaptic ion channels (5, 6). In this report the actions of BTX on conducting and synaptic membrane in the electroplax are examined and found to be limited to conducting membrane.

### MATERIALS AND METHODS

The isolation of BTX, a steroidal alkaloid (molecular weight 538), and the preparation of batrachotoxin methiodide (a quaternary analog of BTX) have been previously described (7). No unreacted BTX was detected in the quaternary preparations. Stock solutions in ethanol were stored at –20°. The monocellular electroplax preparation was essentially the same as that described previously (5, 8).

The effects of various agents were determined by their addition only to the solution bathing the innervated face unless otherwise stated. The composition of the eel saline solution in mM was: NaCl, 188; KCl, 5; CaCl<sub>2</sub>, 2; MgCl<sub>2</sub>, 2; Na<sub>2</sub>HPO<sub>4</sub>, 1.5; glucose, 10. The temperature was 25°. The pH was 7.2 unless otherwise noted, and 2 mM Tris replaced phosphate at pH 8.5.

The electric eels used in this work were supplied locally in Colombia. Most were 5–6 feet long, vigorous, and eating regularly. The action and resting potentials and the maximum depolarization with carbamoylcholine were on the average larger than those seen with eels transported to and maintained for some time in the United States. The metabolic condition of the dissected cells appears to have a strong influence on the characterization of BTX effects, as noted below.

### RESULTS

**Effects of BTX on the Action Potential and the Resting Membrane Potential.** BTX has no effect on the electroplax for periods up to 1 hr unless the cell is stimulated repetitively, i.e., unless the sodium channels of the conducting membrane are activated by action potentials (1). As shown in Fig. 1a, the duration of the action potential increases progressively during a period of stimulation until it reaches a value of several seconds. Only the recovery phase of the action potential is significantly affected; neither the rising phase nor the overshoot is altered.

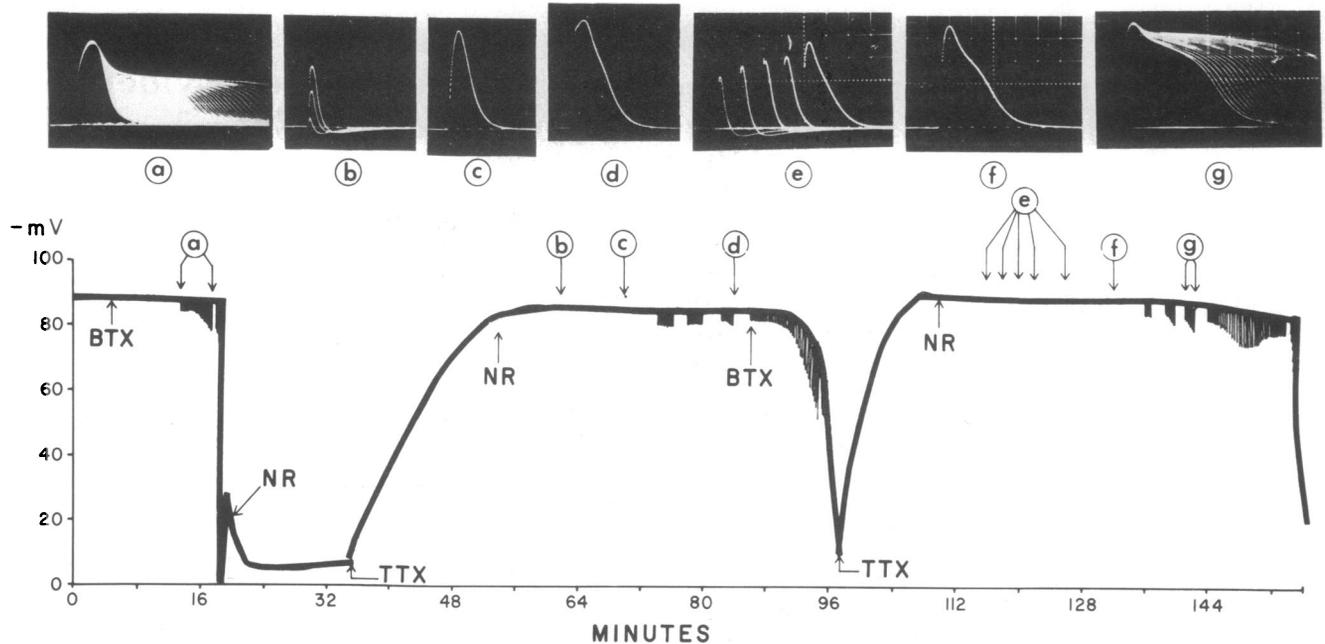


FIG. 1. Interaction of BTX with the innervated face of an electroplax cell. Action potentials were induced by cathodal stimulation. Vertical bars on the membrane potential trace indicate periods of stimulation at a frequency of 1 per sec, a duration of 0.1 msec, and a stimulus strength slightly above threshold. Residual nerve terminals are not stimulated under these conditions, and thus synaptic events are insignificant. BTX, 20 nM in eel saline solution (NR); TTX, 0.60  $\mu$ M. Insets record action potentials at various points in the membrane potential record. Insets *a* and *g* record superimposed action potentials. The amplitude of the initial action potential was 110 mV. Calibrations of oscilloscope grid: inset *a*, 33 mV per vertical division and 2 msec per horizontal division; insets *b-g*, 17 mV and 2 msec.

The action potential in a normal control cell is unaffected by stimulation for at least 30 min.

In the presence of BTX, the prolongation of the action potential resulted in a progressive depolarization of the cell membrane. When the membrane was depolarized by about 15–20 mV, the cell depolarized abruptly and the potential reached a steady state between 0 and –10 mV. All the stimulated cells responded similarly, although the threshold for the abrupt depolarization varied somewhat. The abrupt depolarization phase is all-or-none, shows little dependence on the concentration of BTX, and in some cells remains irreversible for as long as 1 hr in the eel saline solution. The number of stimuli required to induce the sustained all-or-none depolarization, however, does depend on the BTX concentration and on the size of the action potential; the higher the concentration and the larger the action potential, the fewer the stimuli required to depolarize the cell. The lowest BTX concentration that gives this effect at pH 7.0 is about 20 nM.

BTX has no effect when applied solely to the noninnervated face of the electroplax, even when the cell is stimulated extensively.

**Antagonism between BTX and TTX.** TTX reverses the depolarization induced by BTX (see the data following points *a* and *d* in Fig. 1). The rate of repolarization varies somewhat from cell to cell and appears to depend on the metabolic state of the cell and on the length of time the cell is maintained in the depolarized state. In cells with a large action potential (at least 130 mV amplitude) from vigorous eels obtained from and studied in Colombia, 20 nM TTX (a concentration that does not block a control action potential) repolarizes the cell completely within 10 min; in contrast, in cells with smaller action potentials, 5  $\mu$ M TTX may only repolarize by less than 50% within 1 hr. Furthermore, 1 mM ouabain decreases and sometimes prevents the TTX-induced repolarization. These observations suggest that repolarization depends both upon a block by TTX of a BTX-induced  $\text{Na}^+$  conductance increase and upon the activity

of an endogenous  $\text{Na}^+, \text{K}^+$ -ATPase to restore ion concentration gradients.

If TTX is removed by washing before a substantial reversal of the BTX-induced depolarization is achieved, the cell will depolarize spontaneously (Fig. 2). TTX treatment must be maintained until the cell repolarizes to within 20–30 mV of the normal resting potential if the spontaneous depolarization is to be prevented. The steady-state sodium conductance in a BTX-treated cell thus may be a function of the membrane potential.

TTX does not appear to compete directly with BTX at a common binding site. The rate of TTX-induced repolarization is the same in the presence or absence of BTX. Furthermore, if a low concentration of TTX (30 nM) that does not block the action potential and BTX are applied simultaneously, repetitive stimulation fails to induce either a prolongation of the action potential or a depolarization of the cell membrane; when both toxins are washed out with eel saline solution, however, the cell abruptly depolarizes in all-or-none fashion. A similar result has been obtained on rat diaphragm (9). These observations suggest

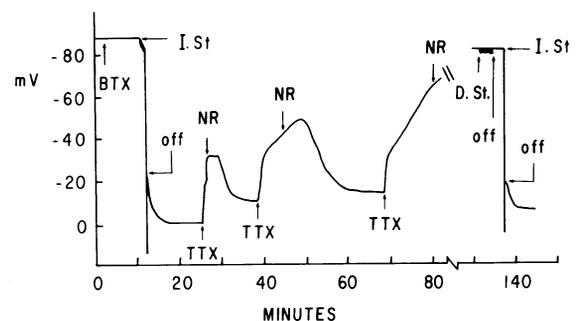


FIG. 2. TTX-induced recovery of the resting potential in a cell treated with BTX. The experimental procedure is the same as for Fig. 1 except BTX is 200 nM; TTX, 6  $\mu$ M. I.St, anodal stimulation; D.St, cathodal stimulation.

that, while TTX does not prevent the access of BTX to the BTX site of action, it does prevent the manifestation of the BTX action. A higher concentration of TTX that blocks the action potential also blocks all effects of BTX, an observation consistent with the necessity of action potential firing for BTX effects.

**Partial Reversibility of BTX.** The BTX effect on the action potential is both cumulative and partially reversible. After washing of a BTX-treated cell, the first action potential of a second train of stimulation is always shorter in duration than the last action potential of the first train. Partial reversibility is also indicated by the sequence of treatments in Fig. 1. After the all-or-none depolarization following *a* in Fig. 1, the cell was washed extensively with eel saline solution for 15 min prior to repolarization with TTX and was then further washed with eel saline solution. Fig. 1*b, c,* and *d* demonstrates a progressive recovery of action potentials which were only slightly prolonged. Despite extensive stimulation the cell did not depolarize again until a second BTX application (Fig. 1*d* and following data). At this point TTX was applied to the depolarized cell without an intervening wash period, and a somewhat faster repolarization led to a progressive recovery of action potentials (Fig. 1*e*), but these action potentials had a longer duration than those of the earlier recovery (compare *e* with *c* and *g* with *d* in Fig. 1). Furthermore, additional stimulation led to a depolarization of the cell without another exposure to BTX (Fig. 1*g* and following data). Apparently the wash period in the initial depolarized state (Fig. 1*a* and following data) more efficiently removed bound BTX than the wash period after the second repolarization (Fig. 1*e, f,* and *g*). In fact, prolonged washing (>90 min) of cells from a particularly vigorous eel in the initial depolarized state gave complete recovery of the resting potential.

**Effect of pH on the Action of BTX and Its Quaternary Analog.** At pH 6.0 BTX (2 nM) has no effect on the action potential during extensive repetitive stimulation. However, when the pH is changed to 8.5 in the presence of the same low BTX concentration, the duration of the action potential increases somewhat but not enough to induce an all-or-none depolarization. Because the tertiary nitrogen in BTX has a  $pK_a$  of 7.4 (2), this pH effect could be explained by a greater membrane solubility of the uncharged species. It is also possible that macromolecular groups that interact with BTX have  $pK_a$ s in this range, which could reduce the interaction at lower pH. During an initial pH 6.0 exposure to 100 nM BTX in Fig. 3, repeated stimulation had no effect on the action potential (Fig. 3*a*). Continued stimulation after the cell had been washed with pH 8.5 eel saline solution resulted in an immediate progressive increase in the duration of the action potential (Fig. 3*b*). When the pH of the eel saline solution bathing the cell was alternated between 6.1 and 8.5, a reversible prolongation of the action potential was observed only at pH 8.5 (Fig. 3*a* to *f*). In Fig. 3*f* the pH was changed from 8.5 to 6.1 during stimulation and the decrease in duration of the action potential can be clearly noted.

Fig. 3 suggests that while BTX remains bound to the cell during both pH 6.1 and 8.5 washing periods, its effect on the action potential occurs only at the higher pH. To test whether deprotonation of the  $pK_a$  7.4 tertiary amine in BTX is responsible for this effect, the quaternary BTX analog that is methylated at this position was investigated. This analog is about 50 to 100 times less potent than BTX on the electroplax, suggesting that the uncharged tertiary amine form either is more active or penetrates the cell membrane more readily. Stimulation of a cell in the presence of the quaternary analog (2  $\mu$ M) at pH 6.2 had no effect on the action potential. However, stimulation in

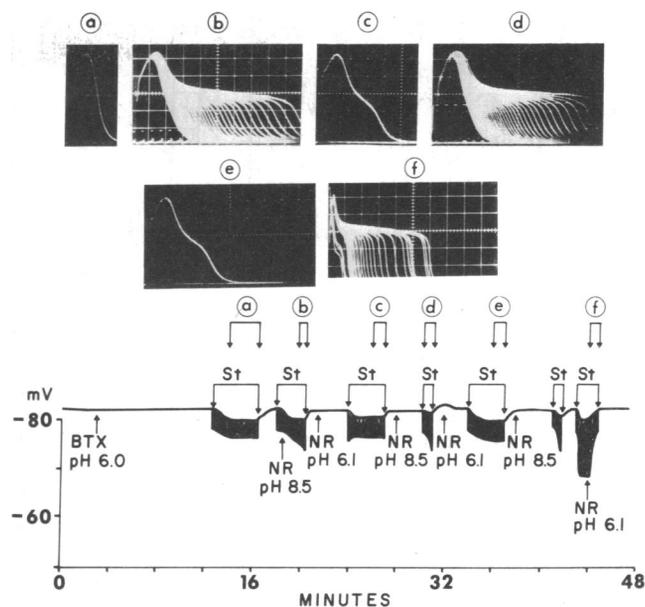


FIG. 3. Effect of pH on the action of BTX. The procedure is the same as for Fig. 1 except BTX is 100 nM. All insets record superimposed action potentials. The initial action potential amplitude was 100 mV. Calibrations: insets *a*–*e*, 17 mV and 2 msec; inset *f*, 17 mV and 5 msec.

the presence of the same analog concentration at pH 8.5 led to an immediate progressive increase in the action potential duration. Because the quaternary analog presumably is unchanged between pH 6.2 and 8.5, these results suggest that cell macromolecular groups that interact with BTX or its quaternary analog have greater efficacy at higher pH. Washing the cell that had been stimulated in the presence of the quaternary analog at pH 8.5 with pH 6.2 eel saline solution did not result in a reduction in duration of the evoked action potentials; the prolonged durations remained constant until the wash pH was changed to 8.5, after which repeated stimulation again gave rise to progressive increases in the durations of the action potentials.

**BTX-Induced All-Or-None Depolarizations of the Cell Membrane Triggered by Other Depolarizing Agents.** Agonists that activate the acetylcholine receptor induce a depolarization of postsynaptic membrane that results in a depolarization of the entire innervated face of an electroplax cell. Such a depolarization induced by carbamoylcholine is not altered by the presence of BTX (50–200 nM), and membrane and action potentials recover normally on washing with eel saline solution even in the presence of BTX. Results in previous sections suggest, however, that BTX gains access to its sites of action only during stimulation. When the application of carbamoylcholine is preceded by a brief exposure to BTX accompanied by stimulation, carbamoylcholine alone can trigger an all-or-none depolarization similar to that obtained by stimulation of a BTX-treated cell. Not only cholinergic agonists but also KCl can trigger an all-or-none depolarization under these conditions. As shown in Fig. 4, the rate of the abrupt depolarization induced by carbamoylcholine or KCl is even greater than that induced by stimulation. For these agents to trigger the all-or-none depolarization, however, they must be added at a concentration sufficient to normally produce a 20–30 mV depolarization in a control cell not treated with BTX.

While triggered by the activation of synaptic ion channels, the all-or-none depolarization elicited by carbamoylcholine in a BTX-treated cell does not appear to proceed through con-

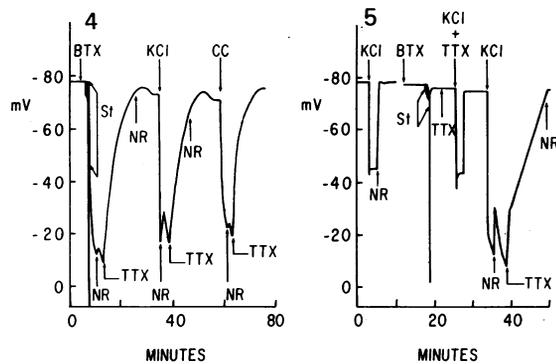


FIG. 4. Triggering of BTX-induced all-or-none depolarization by either carbamoylcholine or  $K^+$ . The procedure is the same as for Fig. 1. BTX is 100 nM; TTX is 100 nM; KCl is 30 mM; and CC is 20  $\mu$ M carbamoylcholine.

FIG. 5. Prevention of the KCl-induced all-or-none depolarization by TTX. Conditions were the same as for Fig. 4. The initial action potential amplitude was 120 mV.

ductance increases in synaptic ion channels controlled by the acetylcholine receptor. Such conductance increases are blocked by the antagonist *d*-tubocurarine, an agent that has no effect on the all-or-none depolarizations induced by stimulation of a BTX-treated cell. With a BTX-treated cell in the presence of 10  $\mu$ M *d*-tubocurarine, 1 mM carbamoylcholine is necessary to reach the 20–30 mV threshold depolarization at which an all-or-none depolarization occurs. This abrupt depolarization occurs with the same rate and to the same extent as that induced by 30  $\mu$ M carbamoylcholine in a BTX-treated cell in the absence of *d*-tubocurarine. Thus, the magnitude of the all-or-none depolarization does not appear to be affected by the presence of *d*-tubocurarine.

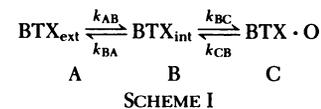
TTX prevents the all-or-none depolarization triggered by either KCl or carbamoylcholine in a BTX-treated cell. When TTX was added together with either KCl (Fig. 5) or carbamoylcholine to such cells, only the depolarization normally induced by either agent in non-BTX-treated cells was observed. The magnitude of the carbamoylcholine depolarization was approximately the same at all pH values between 6.0 and 8.5; in this respect the action of BTX differs from that of veratridine, because veratridine enhances or potentiates carbamoylcholine depolarizations at high pH (5, 6). An additional all-or-none depolarization did occur if the combination of TTX and carbamoylcholine was removed by an eel saline solution wash, but the original membrane potential could be restored by further exposure to TTX. The all-or-none depolarization triggered by carbamoylcholine appeared to be independent of pH between 7 and 8.3.

## DISCUSSION

The present observations on the interaction of BTX with the innervated face of the electroplax are consistent with those on other nerve and muscle cells. For example, sustained depolarizations of the innervated face of the electroplax cell can be induced by BTX, and these depolarizations are reversed by TTX. Because TTX is generally accepted to be a specific antagonist of activated sodium channels<sup>\*\*</sup>, this widely observed

action was crucial in leading to the conclusion that BTX selectively activates sodium channels (2). The electroplax cell is unusual in that stimulation in the presence of BTX is necessary for the depolarization to occur. In many other nerve and muscle preparations, exposure to BTX results in a spontaneous depolarization that is, however, greatly accelerated by stimulation (11, 12).

BTX is known to penetrate slowly to its site of action in a variety of nerve and muscle cells (2, 3, 13), and the requirement for repetitive stimulation for BTX-elicited depolarizations in the electroplax again indicates a slow penetration. The observations on the electroplax can be interpreted most simply in terms of three pools for BTX (Scheme I).  $BTX_{ext}$  is in the



external solution bathing the innervated face.  $BTX_{int}$  refers to an internal phase or phases, perhaps the cell cytoplasm, an internal plasma membrane phase, or a complex with an inactive sodium channel. In  $BTX \cdot O$ , BTX and sodium channels are associated in an active or high sodium conductance state. The rate constants  $k_{XY}$  refer to the rates at which BTX moves from pool X to pool Y. The existence of  $BTX_{int}$  is indicated by the continued effects of BTX in washed, inactive cells. Thus, a TTX-repolarized and washed cell can still manifest a BTX depolarization on further stimulation without further exposure to BTX (Fig. 1g); and a washed cell can show cycles of BTX-induced depolarizing after-potentials (Fig. 3). Because in both these cases  $BTX_{ext}$  is absent, progressive BTX effects reflect a shift from  $BTX_{int}$  to  $BTX \cdot O$ .

The present experiments provide little information about the nature of  $BTX_{int}$ . A report that BTX is more effective when applied internally to the squid giant axon (12) suggests that  $BTX_{int}$  could simply be the interior cell cytoplasm. An attempt to pharmacologically isolate BTX-modified sodium channels in frog nerve fibers with local anesthetics during depolarizing voltage clamping (13) suggests that BTX is specifically associated with inactive sodium channels; thus  $BTX_{int}$  could be highly specific complexes of this type. A third possibility is simply to extend Scheme I by dividing  $BTX_{int}$  into  $BTX_{int'}$ , an interior cell phase, and  $BTX_{int''}$ , a specific complex with inactive sodium channels.

Under certain conditions the action of BTX is partially reversible (compare Fig. 1). However,  $BTX_{int}$  is not depleted by washing the cell at the normal resting potential, indicating that  $k_{BA}$  is small. Previous physiological reports have suggested that BTX action is irreversible and thus that  $k_{BA} = 0$  (14), although a biochemical estimate from sodium flux measurements on neuroblastoma cells indicates a half-time for efflux of BTX of 30 min (3). Because the BTX effects in all instances are progressive and not immediate, the rate of interconversion of  $BTX_{int}$  and  $BTX \cdot O$  ( $k_{BC} + k_{CB}$ ) also appears relatively small. Furthermore,  $k_{BC}$  also appears to be pH dependent (Fig. 3) and strongly voltage dependent. Voltage clamp measurements of steady-state voltage-current relationships in single frog nerve fibers treated with BTX indicate a negative slope in the region around the normal resting potential (13). A similar negative slope region (although of smaller magnitude) is seen in the steady state with veratridine-treated fibers and indicates a voltage-dependent sodium permeability increase as the membrane potential becomes more positive (15). This is equivalent to stating that the rate constant  $k_{BC}$  is significantly increased as the membrane depolarizes below the resting potential.

<sup>\*\*</sup> TTX has recently been shown to block sodium conductance increases induced by grayanotoxin and veratridine in Schwann cells. The sodium channels involved in these phenomena, unlike those described in footnote <sup>†</sup>, appear to show no voltage-dependent conductance changes (10).

This voltage dependence can account for the sustained all-or-none depolarization induced either by stimulation or by the depolarizing agents carbamoylcholine or potassium (Figs. 1, 4, and 5). In a cell with sufficient BTX<sub>int</sub>, depolarization to a threshold value increases  $k_{BC}$  to the point where the inward sodium current drives the cell to an apparent sodium equilibrium potential of 0 to  $-10$  mV<sup>††</sup>. This voltage dependence is manifested in a hysteresis loop of the BTX-treated cell (Fig. 4). Thus potassium or other depolarizing agents give enhanced reductions of the cell membrane potential by triggering the high sodium conductance state BTX-O, which is stable in normal eel saline solution; TTX blocks this high sodium conductance, restores the normal resting potential, and thus shifts BTX-O to BTX<sub>int</sub>, the low sodium conductance state, which also is stable in BTX-treated cells in normal eel saline solution.

The voltage dependence of  $k_{BC}$  can also account for the depolarizing after-potential subsequent to the action potential in BTX-treated electroplax (Figs. 1 and 2). Because of the slow penetration of BTX to its site of action, two classes of sodium channels presumably coexist during stimulation: normal, unmodified channels and modified channels BTX-O. As the membrane depolarizes during the rising phase of the action potential,  $k_{BC}$  increases and shifts BTX to BTX-O; normal sodium inactivation of unmodified channels generates the initial falling phase of the action potential, but  $k_{CB}$  is sufficiently small to limit the reversal of BTX-O induced by the initial falling phase and thus gives rise to the subsequent after-potential. Supporting evidence for this model that the depolarizing after-potential arises from BTX-altered sodium channels was noted in the *Results*, where low concentrations of TTX abolished the after-potential without blocking the action potential.

The voltage dependences of the other rate constants in Scheme I are more difficult to assess from the current data. It was suggested earlier (*Results* section on partial reversibility of BTX) that the rate corresponding to  $k_{BA}$  may be increased when the innervated membrane is depolarized. Because BTX effects on cells with normal resting potentials are observed only following stimulation in the presence of BTX,  $k_{AB}$  appears small at the normal resting potential. During stimulation  $k_{AB}$  thus increases either on depolarization or on specific activation of the sodium channels. Preliminary experiments suggest that  $k_{AB}$  does increase on depolarization; cells simultaneously exposed to BTX and depolarized by elevated  $K^+$  concentrations show depolarization properties similar to, though less pronounced than, those of BTX-treated cells generated by stimulation. The possibility of voltage-dependent chemical reactions involving sodium channels can be extended to other reagents specific for these macromolecules. Catterall *et al.* (16) have recently reported that the binding of scorpion toxin to sodium channels in neuroblastoma is decreased by nearly two orders of magnitude when the cells are depolarized.

The hypothesized three-compartment model of BTX interactions in Scheme I can be applied to cells other than the electroplax. In this context, BTX interactions with the electroplax

would differ from those in most other cells in that the increased sodium permeability in the BTX-treated electroplax at the normal resting potential is insufficient to depolarize the cell spontaneously. This insufficiency could arise from an unusually low value for  $k_{BC}$  in the electroplax, but it could also be due to the high  $Na^+, K^+$ -ATPase-generated outward sodium flux or to the unusually high resting potassium conductance in these cells. During an action potential, the electroplax normally even shows potassium inactivation (17). This decrease in potassium conductance occurs when the innervated membrane is depolarized by 20–30 mV and thus could contribute to the BTX-induced all-or-none depolarizations.

It is noteworthy that the BTX effects summarized by Scheme I are limited to sodium channels in the electrically conducting membrane of the innervated face of the electroplax and do not pertain to ion channels associated with the acetylcholine receptor in postsynaptic membrane. The specificity of BTX thus differs somewhat from that of veratridine, which does enhance synaptic depolarizations at high pH even in the presence of TTX (6).

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<sup>††</sup> The sodium equilibrium potentials in BTX-treated cells appear somewhat more negative than usual, perhaps because of a localized layer containing a high concentration of sodium ions on the interior surface of the excitable membrane in the BTX-treated cells (cf. 13).