SAXITOXIN BLOCKS BATRACHOTOXIN-MODIFIED SODIUM CHANNELS IN THE NODE OF RANVIER IN A VOLTAGE-DEPENDENT MANNER

THOMAS A. RANDO AND GARY R. STRICHARTZ
Anesthesia Research Laboratories, Brigham and Women's Hospital and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT The inhibition by saxitoxin (STX) of single Na channels incorporated into planar lipid bilayers and modified by batrachotoxin (BTX) previously has been shown to be voltage dependent (Krueger, B. K., J. F. Worley, and R. J. French, 1983, *Nature* [*Lond.*], 303:172–175; Moczydlowski, E., S. Hall, S. S. Garber, G. S. Strichartz, and C. Miller, 1984, *J. Gen. Physiol.*, 84:687–704). We tested for such a voltage dependence of STX block of the Na current in voltage-clamped frog nodes of Ranvier. The block by STX of normal Na channels showed no modulation in response to maintained (20 s) changes of the membrane potential or to a train of brief pulses to potentials more positive than the holding potential. However, when the nodal channels were modified by BTX, the train of pulses produced a modulation of the block of the Na current by STX. The modulation of STX block depended on the voltage of the conditioning pulses and this voltage dependence agreed well with that predicted from the single channel studies over the membrane potential range used in those studies. In addition, we found that the voltage dependence of STX block was manifest only at potentials equal to or more positive than required to activate the channels. Most of the apparent differences among data from single channels in bilayers, equilibrium binding studies of STX, and the experiments described here are resolved by the hypotheses that (*a*) STX binding to open channels is voltage dependent, and (*b*) the affinities of STX for closed and inactivated channels are independent of voltage, equal, and less than the open channel affinity at potentials <0 mV. Whether these hypotheses apply to the STX block of all Na channels or just of BTX-modified channels remains to be determined.

INTRODUCTION

The neurotoxins saxitoxin (STX) and tetrodotoxin (TTX) bind to a single site on voltage-dependent Na channels and inhibit the flow of ions through the channels in a variety of nerve and muscle preparations (17). Earlier experiments using either equilibrium binding of radiolabeled toxins or electrophysiological assays had failed to demonstrate any effect of membrane depolarization on the ability of STX or TTX to bind or block Na channels (1, 2, 9, 12). Recently (7, 13, 14), however, several studies of single Na channels incorporated into planar lipid bilayers have shown a striking voltage dependence of the potency of these toxins. The channels were purified from mammalian tissue and were modified by batrachotoxin (BTX) to inhibit their inactivation. Detailed studies by both French et al. (7) and Moczydlowski et al. (14) showed that the voltage dependence of the toxins' equilibrium dissociation constants, K_d, defined by the blockade of individual channels, is due to a voltage dependence of both the association (k_1) and dissociation (k_{-1}) rate constants (where $K_d = k_{-1}/k_1$). The constants k_1 and k_{-1} are defined according to the scheme:

$$\mathrm{STX} + \mathrm{R} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{STX} \cdot \mathrm{R},$$

where R represents the Na channel binding site for STX, and STX · R represents the Na channel blocked by STX. One of the characteristics of the voltage dependence of the binding of these toxins, measured in the planar lipid bilayer, is that the calculated time constants for re-equilibration of binding upon changing from one potential to another are on the order of seconds. This explains why previous electrophysiological studies, using depolarizing pulses of milliseconds' or tens of milliseconds' duration, failed to discover this voltage dependence. It was not clear why this voltage dependence was not manifest in the equilibrium binding experiments, but the results presented here suggest one explanation.

Our aim was to determine whether or not the voltage dependence of STX block of Na channels could be demonstrated in a cellular preparation where Na channels could

be activated with or without BTX present. We chose to study Na currents from the frog node of Ranvier, where the actions of STX and TTX have been extensively studied. In order to test for voltage dependence of STX block, the nodes were voltage-clamped at one membrane potential and then "conditioned," either by a single, sustained voltage step or by a train of brief steps, to a different potential. The modulation of STX block was then assayed by a standard test pulse (also given before the conditioning) to look for a change of the magnitude of the Na current. We found that the STX block of BTX-modified channels, but not unmodified channels, was modulated by such conditioning. Our data are consistent with a model in which STX binds to open Na channels with a voltage-dependent affinity. The model predicts that this property of STX binding would not be observed for unmodified channels with the conditioning protocol used because the open state is so short-lived. However, in BTX-modified channels, the open state persists during a depolarizing step since BTX inhibits Na channel inactivation, and thus the voltage dependence of STX binding is revealed. A preliminary report of a portion of these results has been published (16).

METHODS

Single myelinated fibers were isolated from sciatic nerves of the frog *Rana pipiens* and voltage-clamped according to the method of Dodge and Frankenhaeuser (4). Leakage and capacitance currents were subtracted by use of analog circuitry. The Ringer's solution bathing the node contained 110 mM NaCl, 2.5 mM KCl, 2.0 mM $CaCl_2$, 5 mM HEPES buffer, and 12 mM tetraethylammonium to block the delayed K current. The solution was brought to pH 7.2 with 1 N NaOH. The "intracellular" solution (the solution bathing the cut ends of the fiber) was 110 mM CsCl, 10 mM NaCl, and 5 mM HEPES, pH 7.2. Purified STX was a generous gift of Dr. Sherwood Hall (Food and Drug Administration, Washington, DC). BTX was generously provided by Dr. John Daly (Laboratory of Bio-organic Chemistry, National Institutes of Health, Bethesda, MD). All experiments were performed at room temperature (20–22°C).

MODEL USED FOR PREDICTIONS

The model used to predict the voltage-dependent behavior of STX binding was taken from the studies of Moczydlowski et al. (14), in which the block by STX of single, BTX-activated Na channels incorporated into planar lipid bilayers was studied.

The results from those studies were that the rate constants of STX block of single channels could be described by the following equations:

$$k_1 = 14.0 \times 10^6 \exp(-0.0092 \times E_m)\ \mathrm{M^{-1}\,s^{-1}}$$

and

$$k_{-1} = 0.061 \exp(0.015 \times E_m)\ \mathrm{s^{-1}},$$

where E_m is the membrane potential. These rate constants are assumed to be instantaneous functions of voltage, and thus so is the value of the K_d. The single channel parameters were measured at membrane potentials between −60 and +60 mV, so all predictions beyond this range are extrapolations using the above equations.

The predicted curves in Figs. 1–4 are based on the assumption that STX block of Na channels in the node of Ranvier is identical to that observed in the planar lipid bilayer. The predicted curves in Fig. 5 are based on modifications of this assumption. Essentially, the model predicts the magnitude of the peak Na current (or steady state Na current for BTX-modified channels), which follows the time course of the change of STX occupancy of Na channels {occupancy = [STX]/([STX] + K_d)}. Upon changing from one membrane potential to another, the occupancy is predicted to attain a new equilibrium value (because of the voltage dependence of the K_d) with a voltage-dependent time constant, τ ($\tau = 1/\{(k_1 \cdot [STX]) + k_{-1}\}$).

A detailed mathematical description of the predicted behavior of STX block of Na channels in response to the conditioning protocols used in Figs. 2–5 is presented in the Appendix.

RESULTS

Because of the very long time required for the re-equilibration of STX binding calculated from the single channel studies, we had to use protocols for changing the potential that would keep the membrane depolarized long enough to display any voltage-dependent modulations of the affinity of STX. We used two protocols to test for this modulation. The first is a maintained depolarization that mimics the experimental conditions of the planar bilayer (Fig. 1 *A*). From the holding potential, the membrane is depolarized to a conditioning potential for 20 s. During this time, the occupancy of Na channels should relax toward a new value determined by the value of the K_d at the conditioning potential. This relaxation is an exponential process whose time constant, τ, is also voltage dependent. The membrane is then repolarized to the holding potential and the occupancy should relax back toward the original equilibrium value at a rate determined by the τ at the holding potential. The peak Na current is assayed by brief test depolarizations at various times (t_r) after the beginning of the repolarization.

When 3.0 nM STX was applied to a node, the Na current was reduced to 19% of its control value (3.0 nM STX was predicted from the bilayer model to reduce the Na current to 6% of control at this holding potential). A 20-s depolarization should, according to the parameters from the single channel studies, alleviate the block by STX since the affinity of the toxin for the channel is lower at more positive potentials. Upon repolarization, the block should be restored; the time course of the restoration process is described by the solid curve in Fig. 1 *B*. However, the observation was that the maintained depolarization resulted in none of the predicted modulation of STX block (Fig. 1 *B*, triangles).

The maintained depolarization may yield a poor comparison to the single channel studies, however, since the single channels were modified by BTX and thus were noninactivating, whereas the nodal channels were inactivated for nearly the entire time that the membrane was depolarized. This idea led us to use a second pulsing protocol to test for the voltage dependence of STX block. The second method is a repetitive pulsing procedure that drives the membrane back and forth between the holding potential and a conditioning potential (Fig. 2 *A*). This protocol repeatedly activates the channels and the channels

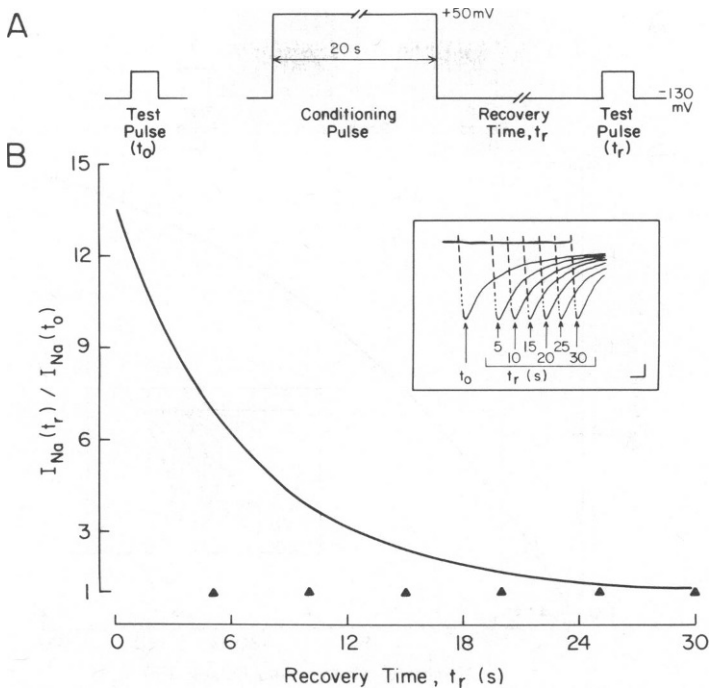

FIGURE 1 The effect of a maintained membrane potential change on the block of Na channels by STX. (*A*) Pulse protocol. We used a 20-s conditioning pulse to +50 mV. During that pulse, the number of channels blocked by STX was predicted to decrease since the K_d for STX is much higher at +50 mV (14.6 nM) compared with that at the holding potential of −130 mV (0.19 nM). After the conditioning pulse, the membrane was returned to the holding potential and the occupancy of the channels was predicted to increase during the recovery time and thus the Na current in response to a test pulse (at t_r) was predicted to decrease. (*B*) All values of Na currents were normalized to that before conditioning (t_o). Solid curve: the predicted decrease of the Na current during the recovery time as described in *A*. Triangles: the observed values of the peak Na current at various times after the conditioning pulse. Inset: actual current traces in response to test pulses before (t_o) and at indicated times after the conditioning pulse. Scales: 0.5 nA, 0.5 ms. N.B. The processes of fast and slow inactivation are not accounted for in the prediction. The time constants for these processes are on the order of milliseconds and hundreds of milliseconds (3, 11), respectively, and control experiments (in the absence of STX) showed that full recovery was achieved by 2–3 s after the conditioning pulse. The first time point tested in the presence of STX was 5 s. We observed no process with a time constant of ultraslow inactivation (6).

are inactivated for a smaller fraction of the total conditioning time. The single channel studies predict that during each pulse cycle the occupancy by STX would be driven first toward its equilibrium value at the conditioning potential and then back toward its equilibrium value at the holding potential, and that it would reach an intermediate, steady state value after a sufficient number of pulses. The modulation of the peak Na current is again assayed by a standard test pulse, but in this protocol the test is applied 90 ms after the final conditioning pulse. The predicted time course of the modulation of the peak Na current by repetitive pulsing in the presence of STX is given by the solid curve in Fig. 2 *B*.

Conditioning with this protocol in the absence of STX resulted in no modulation of the peak Na current. When 1.5 nM STX was applied, the Na current was reduced by 55% (again, less reduction than predicted). Conditioning by pulsing to +50 mV for 10 ms at 10 Hz also resulted in no modulation of the peak Na current (Fig. 2 *B*, triangles), as if there were no voltage dependence to the block of nodal Na channels by STX. Thus, both conditioning protocols failed to demonstrate any influence of membrane potential on the occupancy of nodal Na channels by STX.

An obvious difference between our experimental conditions and those used in the planar bilayer studies is the presence of BTX in the bilayer. The possibility that the observed voltage dependence of STX block of single Na channels was related to the modification by BTX was intriguing. The channel characteristics that are modified by BTX include gating, ion selectivity, and inhibition by local anesthetics, and in fact it was recently stated that "the only channel structure that did not change noticeably in BTX-modified Na channels proved to be the tetrodotoxin receptor" (10).

Nodal currents were partially modified by BTX. After application of 0.2–0.4 μM BTX, the node was stimulated at 10 Hz with brief, large depolarizations (5) for 5 min. After this procedure, the node was washed with Ringer's solution to remove BTX from the bath and there remained a fraction of the Na channel population modified by BTX and an unmodified fraction. Since these different channels are activated in different potential ranges and inactivate at very different rates (10), we could study the two populations independently in the same node.

We investigated the modulation of the BTX-modified Na current by the repetitive pulsing protocol. To do this,

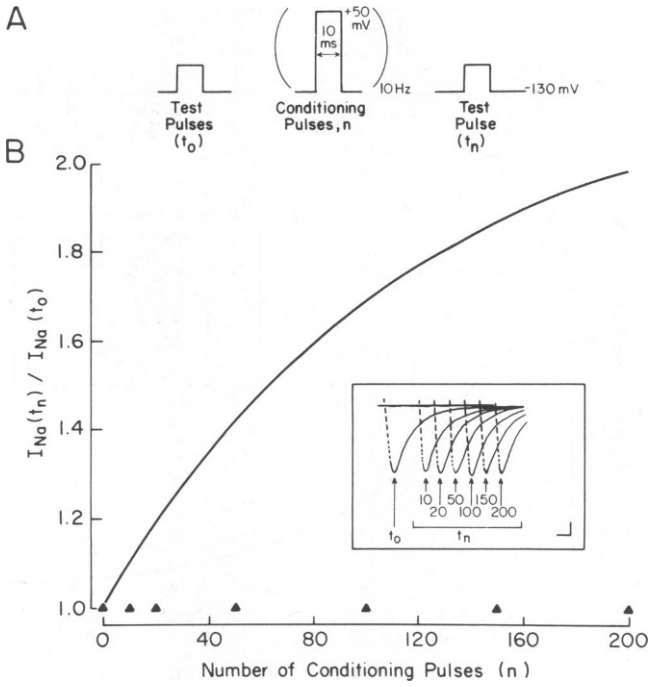

FIGURE 2 The effect of repetitive pulses on the block of Na channels by STX. (*A*) Pulse protocol. We used a high-frequency (10 Hz) train of pulses to modulate the block by STX. According to the single channel data, the block by STX should decrease as the number of conditioning pulses increases since the affinity of the channels for STX decreases during the conditioning pulse. The effect of the conditioning train was assayed by a standard test pulse given 90 ms after the final conditioning pulse. (*B*) The normalization is as in Fig. 1. Solid curve: the predicted increase in the presence of STX as a function of the number of conditioning pulses (see Appendix). Triangles: the observed peak Na currents in response to test pulses (t_n) after various numbers of conditioning pulses. Inset: actual current traces before (t_0) and after the indicated number of conditioning pulses are shown. Scales: 2.0 nA, 0.5 ms.

we conditioned as in Fig. 2 *A*, but gave test pulses to −80 mV, which elicited only BTX-modified Na current. This conditioning had no effect on the magnitude of the BTX-modified Na current in the absence of STX (Fig. 3, circles).

However, the addition of STX altered this response. When 1.5 nM STX was applied to a node modified by BTX, both the unmodified and the modified currents were reduced by ~60%. Repetitive pulses (as in Fig. 2 *A*) resulted in an alleviation of the block of BTX-modified Na current (Fig. 3, triangles), though not precisely as predicted (Fig. 3, solid curve). When our test pulse was to 0 mV, and thus elicited both modified and unmodified currents, the block of only the steady state (BTX-modified) current was significantly modulated by repetitive pulsing. The peak (unmodified) current was slightly increased, but this can be accounted for by the small contribution of BTX-modified current to the peak of the transient current.

The steady state modulation of the Na current that we observed was smaller than predicted with a conditioning potential of +50 mV. We calculated the theoretical steady state values of the Na current after repetitive pulsing as a function of the conditioning potential. This is shown as the solid curve in Fig. 4 *B*. When the conditioning potential was varied experimentally, the steady state values of the modulation of the Na current by STX (Fig. 4 *B*, symbols) were different from the predicted values in three ways. First, before any conditioning, STX was less effective than predicted, as mentioned above (Fig. 4 *B*, arrow). Second, from the single channel studies, it was predicted that any depolarization from the holding potential should result in an alleviation of block. However, we found that the block by STX was actually enhanced with conditioning potentials between −100 and 0 mV. With the parameters used, pulsing to 0 mV produced no change of the block, and only at potentials more positive than 0 mV was there an alleviation of the block. Finally, the single channel studies predicted that pulsing to any potential different from the holding potential should result in a change of the block by STX at steady state. We found that repetitive pulsing to potentials between −150 and −110 mV resulted in no change of the magnitude of the Na current.

The one similarity between the observed and the predicted values of Fig. 4 *B* is that the observed potential dependence of the modulation of STX block was roughly parallel to that predicted between −50 and 100 mV. This suggested to us that, over that potential range, the single channel studies do qualitatively predict the behavior of STX block of BTX-modified nodal Na currents.

It was clear that the single channel predictions over the potential range of −150 to −50 mV were qualitatively inaccurate. The reduction of the BTX-modified Na currents over the voltage range of −150 to −110 mV were

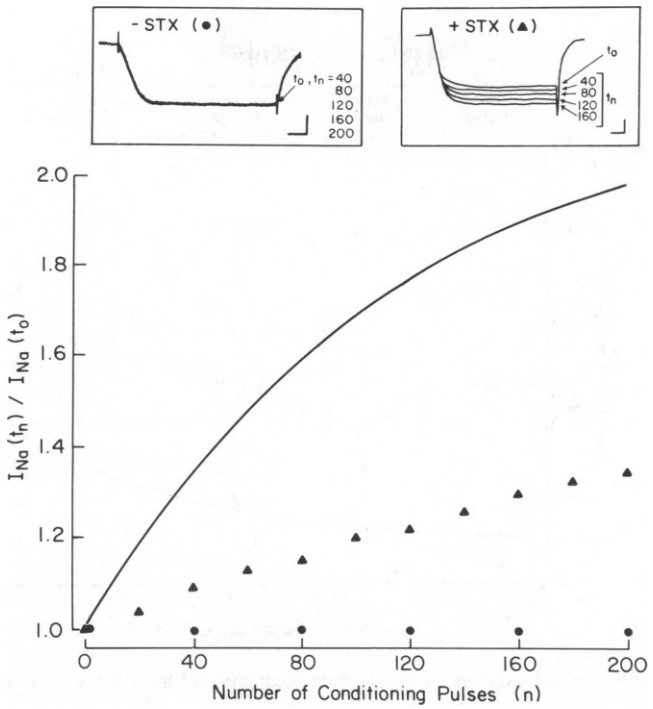

FIGURE 3 The effect of repetitive pulses on the BTX-modified Na current and on the block of BTX-modified Na channels by STX. Before STX application, nodal Na channels were modified by BTX (see text). The pulse protocol was the same as in Fig. 2 *A*, except that we used standard test pulses to −80 mV to elicit only BTX-modified Na current (10). The normalization is as in Fig. 1. Solid curve: the same prediction as in Fig. 2 *B*, because it is based on the same pulse protocol. Circles: the effect of the pulse protocol of Fig. 2 *A* on the BTX-modified current in the absence of STX. Triangles: the observed modulation of the STX block of the BTX-modified Na current as a function of the number of conditioning pulses. Insets: traces of BTX-modified currents before (t_o) and after the indicated numbers of conditioning pulses are shown both in the absence (− STX) and presence (+ STX) of STX. Scales: − STX: 2.0 nA, 1.0 ms; + STX: 1.0 nA, 1.0 ms.

more accurately described by the rate constants of STX binding derived empirically from voltage-clamped frog nodes (8).

These observations led us to the following hypothesis. Closed Na channels bind STX in a voltage-independent fashion, with rate constants as determined from studies of voltage-clamped nodes of Ranvier (8). Open channels bind STX in a voltage-dependent fashion with rate constants as determined from BTX-modified single channels (14). Thus, the STX block of BTX-modified channels would be uniformly voltage dependent at potentials at which all channels are open (greater than −70 mV) and voltage independent at potentials at which all channels are closed (less than −110 mV), and a combination of the two at intermediate potentials.

When this voltage independence of STX binding to closed channels was incorporated into the model to predict the behavior of STX block of Na channels in response to repetitive pulsing (see Appendix), we obtained the dashed curve in Fig. 5 *A*. As can be seen, this curve does not fit the data perfectly, but it does predict qualitatively the behavior of the voltage-dependent modulation of STX block. There is no modulation with conditioning potentials more negative than −110 mV. Conditioning potentials more positive than −110 mV are predicted to result in an enhancement of block but, as the potential becomes more positive, this process reverses, and at potentials more positive than −50 mV, there is an alleviation of block. The major difference between the predicted and observed values is the potential at which there is no change of STX block by repetitive pulsing: 0 mV for the observed, −50 mV for the predicted. In fact, when the voltage dependence of each rate constant was shifted by 50 mV (Fig. 5 *A*, solid line), we obtained a good fit to the data.

Our first impression after the observations in Fig. 3 was that the voltage dependence of STX block was limited to BTX-modified Na channels. However, the more general hypothesis stated above is that the voltage dependence of STX block is limited to open channels and that it was observed only in the presence of BTX because channels are maintained open during a depolarization. When we extend the prediction to nodes not treated with BTX, in which it is assumed that the channels are open on the average for only 0.5 ms during a depolarizing pulse, the predicted steady state modulation of STX block vs. voltage of conditioning pulses is given by the solid curve in Fig. 5 *B*. Only the slightest voltage-dependent modulation is predicted under these circumstances. Therefore, the lack of any apparent voltage dependence of STX block seen in the experiments without BTX may be due to the fact that the channels were

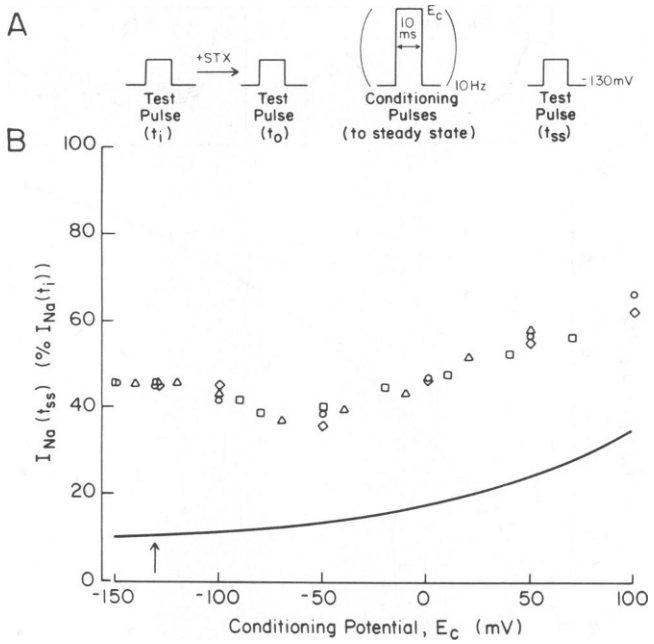

FIGURE 4 The effect of the conditioning potential on the steady state modulation of STX block of BTX-modified channels in response to repetitive pulses. (*A*) Pulse protocol. This protocol was similar to that in Fig. 2 *A* except that we used enough conditioning pulses (300 were sufficient at all conditioning potentials) for the binding of STX to reach a steady state and again used test pulses to −80 mV to elicit only BTX-modified Na current. (*B*) The currents in response to the test pulses at steady state (t_{ss}) were normalized to the current before STX application (t_i) rather than to that before conditioning (t_o) as in previous figures. Solid curve: the predicted relationship between the steady state reduction of Na current by 1.5 nM STX and the potential of the conditioning pulses (see Appendix). Symbols: data from four experiments showing the modulation of the STX block of BTX-modified Na currents as a function of the conditioning pulse potential. The points from the individual experiments, all with 1.5 nM STX, were normalized such that the fractional blocks before conditioning (at t_o) are equal. Thus, each set of points was multiplied by a constant: triangles, 1.05; circles, 1.21; diamonds, 0.86; squares, 1.10. Arrow: the predicted and observed values at this potential correspond to the Na current in response to the test pulse before conditioning, t_o (because it is equivalent to the current at t_{ss} when E_C = the holding potential).

open for too small a fraction of the time at the conditioning potential for the binding of toxin molecules to have responded.

DISCUSSION

We have demonstrated that STX block of nodal Na channels is voltage dependent, but that the voltage dependence is manifest only when the Na channels are modified by BTX. There are two hypotheses that can be distinguished experimentally to account for the results. The first is that the voltage dependence of STX binding is a direct effect of BTX. That is, along with many other channel modifications conferred by BTX, perhaps the binding of this (and other?) alkaloid neurotoxin(s) induces a new conformation of the STX/TTX binding site that is modulated by membrane potential. A second possibility is that the voltage dependence of STX binding is a property of the open channel conformation in general. Then the results presented here could be explained by the fact that BTX inhibits Na channel inactivation and the voltage dependence of STX binding is revealed since the channels are open for the full duration of each conditioning pulse.

If the voltage dependence of STX binding is simply a property of open Na channels, then this should be demonstrable experimentally. First of all, other inhibitors of Na channel inactivation, such as *N*-bromoacetamide or chloramine-T (15, 18), should expose this property of STX binding as well. However, the relationship between steady state block and conditioning potential (Fig. 5 *A*, solid curve) should be different from that with BTX since the voltage dependence of Na channel activation would not be shifted as with BTX. There should be no modulation of STX block with conditioning potentials more negative than about −50 mV. A second experiment that would distinguish between the two hypotheses would be to test further for the voltage dependence of STX binding to unmodified channels. If this truly is a property of open channels and is not unique to BTX-modified channels, then in response to more frequent opening of unmodified channels, the voltage-dependent modulation would be enhanced. Using the pulse protocol in Fig. 4 *A*, unmodified channels are predicted to show only the slightest voltage dependence of STX binding, even at very positive potentials (Fig. 5 *B*, solid curve). However, if the frequency of pulsing is increased to 20, 50, and 100 Hz, the voltage-dependent modulation is predicted to become more and more obvious. If this result is observed, it would rule out the possibility that STX binds in a voltage-dependent manner only to BTX-modified channels. These experiments are currently being conducted in our laboratory.

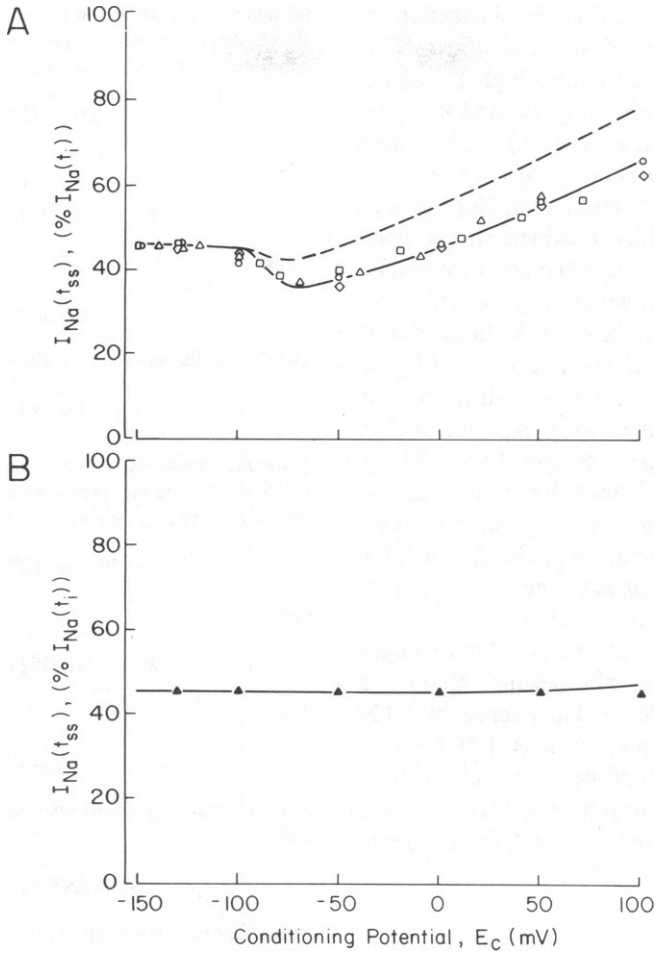

FIGURE 5 Adjustments of the model to fit the data. (*A*) The model used to predict the voltage dependence of STX block of Na channels (see Appendix) was adjusted to fit the data from BTX-modified channels in Fig. 4. Dashed curve: the only modification was that closed channels were assumed to bind STX with the voltage-independent rate constants. Solid curve: in addition to voltage-independent block of closed channels, the model was further modified by shifting the voltage-dependent rate constants of STX binding to open channels by ~50 mV. Symbols: the same data as in Fig. 4 *B*. (*B*) Solid curve: when the model used to predict the solid curve in Fig. 5 *A* was used to predict the expected modulation of STX block in the absence of BTX (see Appendix), the relationship was essentially flat across this voltage range. Triangles: these points demonstrate that the pulsing protocol used in Fig. 4 *A* resulted in no modulation of the STX block of channels not modified by BTX. All points were multipled by a factor of 1.02 to normalize the resting block to that predicted by the model.

The single channel studies permit the direct calculation of the association and dissociation rate constants of STX binding, and thus the calculation of the time constants of re-equilibration of toxin binding at different voltages. These time constants range from 15.1 s at −100 mV to 3.6 s at +100 mV in the presence of 1.5 nM STX. The data in Fig. 3 are consistent with a process whose time constant is on the order of many seconds. It is clear why this voltage-dependent modulation of STX binding was not observed when nodal Na currents were tested with brief (several milliseconds in duration), infrequent pulses, even in the presence of BTX (9, 10).

The results presented here may reconcile an apparent discrepancy between the single channel studies and previous studies of the effect of membrane potential on the binding of STX or TTX. Almers and Levinson (1), studying TTX binding to frog muscle, and Krueger et al. (12), studying STX binding to rat brain synaptosomes, both showed that K^+-induced depolarization of the membranes had no effect on the K_d for toxin binding. This lack of effect would be expected from our results. Whether the membranes maintained normal resting potentials or were depolarized, nearly all Na channels would be either closed or inactivated and would thus bind STX in a voltage-independent manner.

Catterall et al. (2), studying STX binding to rat brain synaptosomes, observed a small inhibition of STX binding when the synaptosomes were depolarized by a high K^+ concentration. Again, all channels would be inactivated under such conditions and no change of STX binding would be expected. They attributed the small inhibition to the known inhibition of STX binding by K^+ compared with choline, which was the predominant cation in their control conditions (19).

Krueger et al. (12) also found that BTX-induced depolarization of the synaptosomes did not alter the K_d for STX

binding. Catterall et al. (2) found that depolarization by high K^+ in the presence of BTX did not affect STX binding any more than depolarization by high K^+ alone. They also found that STX binding to lysed synaptosomes was similar in the presence and absence of BTX. These results may at first appear to be at odds with our findings, but on closer inspection they are consistent. Both groups used BTX, which should stabilize channels in the open state and thus depolarize the synaptosomes completely. Thus, the membrane potential at which they were measuring STX binding would have been near 0 mV. In the model that fits our data closely (Fig. 5 *B*, solid curve), the K_d for STX binding to open channels at 0 mV is identical to that for STX binding to closed channels. Any experiment that both maintains channels in an open configuration and fully depolarizes the membrane would, according to our results, show no change of the equilibrium binding characteristics of STX. We would expect modulation of the K_d for STX binding to BTX-treated membranes at membrane potentials between the resting potential and 0 mV.

Therefore, previous experimental results of binding may differ from single channel data not because there are inherent differences in the voltage dependence of STX binding between the different preparations, but because the voltage-dependent action is a property only of the open state. It will be interesting to determine whether this state dependence can be demonstrated at the level of single channel recordings.

APPENDIX

The purpose of this appendix is to present the mathematical formalism used to derive the predicted curves of Figs. 2–5. Many of the equations from the text are repeated here for clarity. The predictions are based on the assumption that STX binds to nodal Na channels with the voltage-dependent affinity that has been observed for STX block of single Na channels in planar lipid bilayers (see text). The essence of the following equations is to describe the modulation of the STX block of the macroscopic Na current as the membrane is pulsed repetitively from a holding potential to a conditioning potential and back (see Fig. 2 *A*).

Assuming that STX binds to Na channels in a one-to-one stoichiometry (9), the occupancy (B) by STX is given by the following equation:

$$B = [\mathrm{STX}]/([\mathrm{STX}] + K_d), \tag{1}$$

where

$$K_d = k_{-1}/k_1 \tag{2}$$

and k_1 is the association rate constant for STX and k_{-1} is the dissociation rate constant for STX. Occupancy of Na channels by STX is reflected in the magnitude of the peak Na current, I_{Na} (or steady state Na current for noninactivating channels) according to the equation:

$$I_{Na} = (1 - B) \cdot 100\%, \tag{3}$$

so as B increases linearly from 0 to 1, I_{Na} decreases linearly from 100 to 0%. When I_{Na} attains a new value, I'_{Na}, because of a change of B to a new value, B', the normalized change of I_{Na} is given by

$$I'_{Na}/I_{Na} = (1 - B')/(1 - B). \tag{4}$$

B is predicted to change with membrane potential according to the voltage dependence of the K_d as reported by Moczydlowski et al. (14). When the membrane is held at the holding potential, E_H, the rate constants are k_{1H} and k_{-1H} where

$$k_{1H} = (14.0 \times 10^6) \exp(-0.0092 \times E_H) \tag{5}$$

and

$$k_{-1H} = (0.061) \exp(0.015 \times E_H). \tag{6}$$

Thus,

$$K_{dH} = k_{-1H}/k_{1H}, \tag{7}$$

and the equilibrium value for the occupancy at E_H, B_H, is given by

$$B_H = [\mathrm{STX}]/([\mathrm{STX}] + K_{dH}). \tag{8}$$

If the membrane were then to be immediately driven to a conditioning potential, E_C, the rate constants would instantaneously assume the new values, k_{1C} and k_{-1C}, where

$$k_{1C} = (14.0 \times 10^6) \exp(-0.0092 \times E_C) \tag{9}$$

and

$$k_{-1C} = (0.061) \exp(0.015 \times E_C). \tag{10}$$

Thus,

$$K_{dC} = k_{-1C}/k_{1C} \tag{11}$$

and the occupancy would relax toward B_C, its equilibrium value at E_C, where

$$B_C = [\mathrm{STX}]/([\mathrm{STX}] + K_{dC}), \tag{12}$$

with a time constant τ_C given by

$$\tau_C = 1/\{(k_{1C} \cdot [\mathrm{STX}]) + k_{-1C}\}. \tag{13}$$

When the membrane was returned to E_H, the rate constants would again assume the values k_{1H} and k_{-1H}, and the occupancy would relax back toward B_H with a time constant

$$\tau_H = 1/\{(k_{1H} \cdot [\mathrm{STX}]) + k_{-1H}\}. \tag{14}$$

During a single pulse cycle (beginning at E_H, one depolarizing pulse to E_C, then one repolarizing pulse back to E_H), the occupancy would thus be expected to change depending on the values E_H, E_C, [STX], and the durations of the depolarizing and repolarizing pulses. The repetitive pulsing paradigm can be symbolized by the following mathematical scheme.

If a parameter s varies between 0 and 1 as B varies between B_H and B_C, during the first depolarizing pulse (duration = t_C), s rises from its initial value of 0 to some value s_1 with a time constant τ_C. During the subsequent repolarization (duration = t_H), s relaxes back toward 0 to a new value s'_1 with a time constant τ_H. During the second depolarizing pulse, s attains a new value, s_2, and relaxes to s'_2 during the repolarization phase. After n pulses, s will have a value s_n, given by

$$s_n = s_1 \cdot [1 + k + k^2 + k^3 + \ldots + k^{(n-1)}], \tag{15}$$

where

$$s_1 = 1 - \exp(-t_C/\tau_C) \tag{16}$$

and

$$k = \exp(-t_C/\tau_C) \cdot \exp(-t_H/\tau_H). \tag{17}$$

To convert this to occupancy, B is initially B_H (i.e., at t_o). After n pulses (i.e., at t_n), the occupancy B_n is

$$B_n = B_H - [(B_H - B_C) \cdot s_n] \quad (18)$$

and

$$I_{Na}(t_n)/I_{Na}(t_o) = (1 - B_n)/(1 - B_H) \quad (19)$$

(after Eq. 4), where $I_{Na}(t_n)$ and $I_{Na}(t_o)$ refer to the magnitudes of the Na current after n conditioning pulses and before any pulses, respectively. The predicted curves in Figs. 2 and 3 plot the value $I_{Na}(t_n)/I_{Na}(t_o)$ as a function of the number of conditioning pulses. These curves are thus not continuous tracings of normalized I_{Na} during the pulsing cycle. Rather, they are smooth curves through the discrete points, which are the predictions of the normalized I_{Na} only at the end of each depolarizing pulse. The excursions of I_{Na} during the pulsing cycle are so minute under the experimental conditions that the continuous curve and the smoothed curve would barely be distinguishable and would, of course, intersect during each cycle. There is a small error introduced in the prediction by this smoothed curve, but by the same argument it is minuscule. Since I_{Na} is assayed by a test pulse at the end of a repolarizing pulse rather than at the end of a depolarizing pulse, it would be more accurate to calculate the normalized I_{Na} with s'_n values than with s_n values. The resulting smoothed curves would lie below the curves of Figs. 2 and 3, but <0.01 normalized units below, and thus they are insignificantly different.

Figs. 4 and 5 plot steady state values of I_{Na} as a function of conditioning potential rather than a time course of change of I_{Na} with conditioning. Thus, we need a way of calculating the steady state value of the occupancy with repetitive pulsing. Since $k < 1$, the infinite series of Eq. 15 converges to $(1 - k)^{-1}$ and the steady state value of s (i.e., as $n \to \infty$) is given by

$$s_\infty = s_1 \cdot (1 - k)^{-1}. \quad (20)$$

At steady state (i.e., at t_{ss}), the occupancy B_∞ is

$$B_\infty = B_H - [(B_H - B_C) \cdot s_\infty] \quad (21)$$

and I_{Na} (not normalized, but expressed as in Eq. 3) is

$$I_{Na}(t_{ss}) = (1 - B_\infty) \cdot 100\%. \quad (22)$$

As in Figs. 2 and 3, there is a small error in the curves in Figs. 4 and 5 because we calculate the value of I_{Na} at s_∞ and not at s'_∞ (i.e., at the end of the repolarization phase at steady state), but again this error is very small (<1%).

Modifications of the Predictions to Fit the Observations

In Fig. 5, the steady state STX occupancy is assumed to follow the voltage-dependent model only over the potential range where channels are open. Thus, the rate constants of STX binding at the holding potential (Eqs. 5 and 6), at which all channels are closed, are replaced by the voltage-independent rate constants

$$k_{1H} = 6.3 \times 10^6\ M^{-1}\ s^{-1} \quad (23)$$

and

$$k_{-1H} = 0.0079\ s^{-1}, \quad (24)$$

taken from Hahin and Strichartz (8). The binding of STX to open channels is still governed by Eqs. 9 and 10, but in the calculation of B_n (Eq. 18), B_C is replaced by B_{GNa} where

$$B_{GNa} = (G_{Na} \cdot B_C) + [(1 - G_{Na}) \cdot B_H] \quad (25)$$

and

$$G_{Na} = \frac{1}{1 + \exp[(-87 - E_C)/5.16]}. \quad (26)$$

Eq. 26, taken from Dubois et al., Table II (5), describes the activation of BTX-modified Na channels, or the fraction of channels open at the membrane potential E_C. It is a sigmoid curve and gives the relationship of G_{Na} vs. E_C with -87 mV as the midpoint voltage ($G_{Na} = 0.5$) and 5.16 mV as the slope factor. The G_{Na} factor varies between 0.05 and 0.95 from $E_C = -100$ mV to $E_C = -70$ mV.

Thus, by modifying B_C to B_{GNa} by Eq. 25, we have taken into account the hypothesis that the rate constants for STX binding to closed channels are described by Eqs. 23 and 24, whereas those for STX binding to open channels are described by Eqs. 9 and 10. When few channels are opened by pulsing to E_C (e.g., if E_C is around -100 mV), then the right side of the sum of Eq. 25 dominates (since $G_{Na} \to 0$) and $B_{GNa} \to B_H$. In this case, repetitive pulsing results in very little change of B (since $B = B_H$ initially). When nearly all channels are opened by pulsing to E_C (e.g., if E_C is more positive than -70 mV), then the left side of the sum of Eq. 25 dominates (since $G_{Na} \to 1$) and $B_{GNa} \to B_C$. In this case, repetitive pulsing results in a change of B that is determined by B_C and B_H as in Eq. 18, although B_H now has a value that is voltage independent.

These modifications resulted in the prediction shown by the dashed curve in Fig. 5 *A*. The prediction shown by the solid curve in that figure, which fits the data very well, was obtained by shifting the voltage-dependent rate constants of STX binding to open channels by ~50 mV. Thus, Eqs. 9 and 10 were modified to

$$k_{1C} = (14.0 \times 10^6) \exp[-0.0092 \times (E_C - 51.6)] \quad (27)$$

and

$$k_{-1C} = (0.061) \exp[0.015 \times (E_C - 51.6)]. \quad (28)$$

The prediction shown by the solid curve in Fig. 5 *B* was calculated like that in Fig. 5 *A* with two differences, both related to the fact that the prediction in Fig. 5 *B* is for channels not modified by BTX. The first difference is that the value of t_C used in Eqs. 16 and 17 was 0.5 ms instead of 10 ms (see text). The second difference is that the G_{Na} factor (Eq. 26) describes the activation of unmodified channels and thus has a midpoint voltage of -31 mV and a slope factor of 5.46 mV (estimated from our data).

We thank Mr. Richard Roscoe for assistance with computer programming. This investigation was supported by U.S. Public Health Service (USPHS) National Research Service Award 2T 32 GM07753-06 from the National Institutes of General Medical Sciences to T.A.R. and USPHS National Institutes of Health GM15904 to G.R. Strichartz.

Received for publication 21 March 1985 and in final form 10 October 1985.

REFERENCES

1. Almers, W., and S. R. Levinson. 1975. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. *J. Physiol. (Lond.).* 247:483–509.
2. Catterall, W. A., C. S. Morrow, and R. P. Hartshorne. 1979. Neurotoxin binding to receptor sites associated with voltage-sensitive sodium channels in intact, lysed, and detergent-solublized brain membranes. *J. Biol. Chem.* 254:11379–11387.
3. Chiu, S. Y. 1977. Inactivation of sodium channels: second order kinetics in myelianted nerve. *J. Physiol. (Lond.).* 273:573–596.
4. Dodge, F. A., and B. Frankenhaeuser. 1958. Membrane currents in isolated frog nerve fiber under voltage clamp conditions. *J. Physiol. (Lond.).* 143:76–90.
5. Dubois, J. M., M. F. Schneider, and B. I. Khodorov. 1983. Voltage

dependence of intramembrane charge movement and conductance activation of BTX-modified Na^+ channels in frog node of Ranvier. *J. Gen. Physiol.* 81:829–844.

6. Fox, J. M. 1976. Ultra-slow inactivation of the ionic currents through the membrane of myelinated nerve. *Biochim. Biophys. Acta.* 426:232–244.
7. French, R. J., J. F. Worley, and B. K. Krueger. 1984. Voltage-dependent block by saxitoxin of sodium channels incorporated into planar lipid bilayers. *Biophys. J.* 45:301–310.
8. Hahin, R., and G. R. Strichartz. 1981. Effects of deuterium oxide on the rate and dissociation constants for STX and TTX action. *J. Gen. Physiol.* 78:113–139.
9. Hille, B. 1968. Pharmacological modification of the sodium channels of frog nerve. *J. Gen. Physiol.* 51:199–219.
10. Khodorov, B. I. 1983. Modifications of voltage-sensitive sodium channels by batrachotoxin. *In* Structure and Function of Excitable Cells. D.C. Chang, I. Tasaki, W.J. Adelman, Jr., and H.R. Leuchtag, editors. Plenum Publishing Corp., New York. 281–303.
11. Khodorov, B., L. Shishkova, E. Peganov, and S. Revenko. 1976. Inhibition of sodium currents in frog Ranvier node treated with local anesthetics: role of slow inactivation. *Biochim. Biophys. Acta.* 433:409–435.
12. Krueger, B. K., R. W. Ratzlaff, G. R. Strichartz, and M. P. Blaustein. 1979. Saxitoxin binding to synaptosomes, membranes, and solublized binding sites from rat brain. *J. Membr. Biol.* 50:287–310.
13. Krueger, B. K., J. F. Worley, and R. J. French. 1983. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. *Nature (Lond.).* 303:172–175.
14. Moczydlowski, E., S. Hall, S. S. Garber, G. S. Strichartz, and C. Miller. 1984. Voltage-dependent blockade of muscle Na^+ channels by guanidinium toxins. Effect of toxin charge. *J. Gen. Physiol.* 84:687–704.
15. Oxford, G. S., C. H. Wu, and T. Narahashi. 1978. Removal of sodium channel inactivation in squid giant axon by *N*-bromoacetamide. *J. Gen. Physiol.* 71:227–247.
16. Rando, T. A., and G. R. Strichartz. 1985. Saxitoxin inhibition of sodium currents in amphibian nerve is independent of membrane potential. *Biophys. J.* 47 (2, Pt. 2):438*a*. (Abstr.)
17. Ritchie, J. M., and R. B. Rogart. 1977. The binding of STX and TTX to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.* 79:1–50.
18. Wang, G. K. 1984. Irreversible modification of sodium channel inactivation in toad myelinated nerve fibers by the oxidant chloramine-T. *J. Physiol. (Lond.).* 346:127–141.
19. Weigele, J. B., and R. L. Barchi. 1978. Saxitoxin binding to the mammalian sodium channel: competition by monovalent and divalent cations. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 95:49–53.