

Batrachotoxin modifies the gating kinetics of sodium channels in internally perfused neuroblastoma cells

(slow inactivation/fast inactivation/voltage clamp)

LI-YEN MAE HUANG, NAVA MORAN, AND GERALD EHRENSTEIN

Laboratory of Biophysics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205

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ABSTRACT We have studied the effects of batrachotoxin (BTX) on sodium channels in hybrid mouse neuroblastoma cells NG108-15 by using the suction pipet voltage clamp method. BTX-modified sodium channels activate with first-order kinetics and, over most of the potential range, activate more slowly than normal sodium channels. The peak conductance–voltage curve and the time constant of activation-versus-voltage curve for BTX-modified sodium channels are shifted about 50 mV in the hyperpolarizing direction compared to the corresponding curves for normal sodium channels. There is no change in the slope of the conductance–voltage curve. These results suggest that BTX slows down one of the steps leading to channel opening, which consequently becomes rate-limiting. In addition, BTX eliminates both fast and slow inactivation.

Neurotoxins have played an important role in understanding the molecular properties of Na channels in excitable membranes. They have been used to identify the various conductances (1, 2) as well as to purify the molecular components of Na channels (3, 4). Among various toxins studied, batrachotoxin (BTX) is one of the most potent and specific activators of Na channels (5). BTX modifies the activation and inactivation of Na channels, causing them to remain open at the resting membrane potential (6–9). This specific property of BTX is the basis for the isotope flux method used in measuring the permeability change caused by activation of Na channels. Isotope flux experiments and radioligand binding assays carried out in cultured neuroblastoma cells indicate that BTX binds to the same receptor site as several other alkaloid neurotoxins—veratridine, aconitine, and grayanotoxin (10)—but the BTX receptor site is distinct from the receptor site for tetrodotoxin (11). Although these biochemical methods for studying Na channels have yielded much interesting information, they have poor time resolution and limited voltage control. It therefore is particularly important to have a detailed voltage clamp analysis of neurotoxin action in these cultured cells in order to provide direct correlation between the voltage-dependent aspects of Na channel function and biochemical information on the channel structure.

Khodorov (9) has reported the effect of BTX on the Na channels of frog node under voltage clamp. In this paper, we report the voltage-dependent action of BTX on Na channels of internally perfused neuroblastoma cells. We also extend previous work by determining the properties of BTX-modified channels for saturating concentrations of BTX. This avoids ambiguities and inaccuracies that might arise in resolving voltage-clamp records of mixtures of normal and BTX-modified channels into two components. Additionally, we extend previous work by explicitly determining the effect of BTX on slow sodium inactivation.

Preliminary results of these studies have appeared in an abstract (12).

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METHODS AND MATERIALS

Materials. The media and chemicals used were obtained from the following sources: Dulbecco–Vogt modification of Eagle's minimal essential medium and newborn calf serum, GIBCO; Dulbecco's phosphate-buffered saline, Media Unit of the National Institutes of Health; recrystallized trypsin, Worthington. BTX was kindly supplied by J. Daly (Laboratory of Digestive Diseases, National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases).

Selection of the Cell Line. The cell line we used in this work is NG108-15 hybrid neuroblastoma. In the past, neuroblastoma N18 cells have been used extensively to study membrane excitability. Although biochemical and pharmacological information on neurotoxin action on Na channels is available for this cell line, the cells are quite fragile and smaller (15–20 μm in diameter) than NG108-15. Furthermore, the Na^+ current of N18 is usually small (<5 nA) and the cells seldom last more than 30 min under voltage-clamp conditions. NG108-15 cells are larger and have a large stable Na^+ current (>10 nA). The Na channel kinetics of NG108-15 cells are similar to those of N18 cells (unpublished data).

Cell Culture. NG108-15 is a neuroblastoma–glioma hybrid line derived by fusion of mouse neuroblastoma clone N18TG2 (13) with rat glioma clone C6BU-1 (14). Stock cultures of NG108-15 are grown in Dulbecco's modified Eagle's medium containing 5% newborn calf serum, 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine at 36°C in a humidified atmosphere of 10% CO_2 /90% air. To induce neuronal differentiation, cells used for experiments were cultured in this medium, to which 1 μM prostaglandin E_1 and 1 mM theophylline were added for 7–10 days before use. Cells treated in this way are larger in size (30–50 μm in diameter) and more electrically excitable than are the undifferentiated cells.

Electrophysiology. The culture dish was mounted on the stage of a phase-contrast microscope. Unless noted specifically, experiments were performed at room temperature (20–23°C). The external solution contained 130 mM NaCl, 0.8 mM MgCl_2 , 5.4 mM KCl, and 25 mM Hepes. To eliminate K^+ , Ca^{2+} , and Ca^{2+} -dependent K^+ currents, 30 mM tetraethylammonium and 0.5 mM Ca^{2+} were used. The pH of the external solution was adjusted to 7.4 with 14 mM Tris base. The internal solution contained 150 mM CsF, 25 mM Hepes, and 1 mM Na_2HPO_4 , and its pH was adjusted to 7.2–7.3 with 1 M KOH and 1 M NaOH. The final concentrations of internal K^+ and Na^+ were 5–6 mM.

The voltage-clamp circuit was similar to circuits used for experiments on isolated helix neurons (15). The patch electrode was fabricated according to the method of Lee *et al.* (15, 16). Its tip diameter was 10–20 μm , and its resistance was 0.5–0.8 M Ω . The procedure for recording the electrical activity was similar to that described by Lee *et al.* (16). The membrane potential was recorded by a calomel half-cell placed in the suction

Abbreviation: BTX, batrachotoxin.

pipet. A 100- μm -diameter platinum wire coated with platinum black was also placed in the suction pipet for applying current intracellularly. The reference electrode, a calomel half-cell, was connected to the bath by an agar bridge. The cells were internally perfused with a solution flowing through an inlet tube connected to a reservoir located 50 cm above the set-up. Gentle suction was applied through a trap bottle.

After the system was ready, the pipet was brought close to the cell and suction was turned on. The cell was sucked onto the tip of the pipet, and the membrane was ruptured by increasing the suction. The membrane potential usually ranged from -20 to -60 mV. Action potentials could be elicited with constant-current stimuli.

There are three major advantages of this method. First, it uses a low-resistance current-delivering electrode (<200 K Ω compared to 5–10 M Ω for a microelectrode). This greatly improves the speed of voltage clamp, and hence allows clear resolution of Na⁺ current. Second, because the cells are internally dialyzed, the internal ionic composition can be controlled. This is especially important with BTX. Because BTX eliminates inactivation of the Na⁺ current, and because the cells have a small volume, frequent or long depolarizing pulses without dialysis would lead to an accumulation of Na⁺ inside the cells. This would result in continuous changes in reversal potential and errors in the measured Na⁺ current. Third, the cells remain viable longer with this method than with the conventional microelectrode voltage clamp. The Na⁺ currents we tested usually showed little deterioration within 45–60 min after the start of an experiment.

One important parameter which influences the fidelity of the clamp is the series resistance (R_s). The actual R_s is larger than the resistance of the suction pipet measured in the bath because cytoplasmic material is brought into the pipet when the cell is sucked onto it. R_s is usually in the range 500 K Ω –1 M Ω . The measured membrane capacitance ranged from 200 to 400 pF. This value is considerably larger than the capacitance expected for cells of this size, in agreement with the observation that these cells have numerous microvilli (M. Daniels, personal communication). We compensated R_s electronically by means of positive feedback circuitry. After compensation, the time constant of the current transient was approximately 200 μs .

RESULTS

Time Course of BTX Effect. Neuroblastoma NG108-15 cells have a resting potential of -20 to -60 mV depending on the age of the cells and the conditions of growth. When the clamp potential was stepped from the holding potential to a level more positive than -60 mV, the Na⁺ current activated rapidly and then inactivated (Fig. 1*a*).

After BTX was added, the resting potential depolarized to zero. Under voltage clamp, the peak Na⁺ current gradually decreased in amplitude and the steady-state inward current gradually increased in amplitude. The time constant for inactivation, as measured after subtracting the steady-state current, did not change significantly. These observations support the view that there are two populations of channels—normal and BTX-modified. When a saturating concentration of BTX (5 μM) was used, the voltage-clamp records reached steady state about 30 min after BTX application and indicated that BTX-modified channels do not inactivate at all (Fig. 1*b*). Even for pulses as long as 50 sec (not shown), no inactivation was observed, consistent with the depolarization of the resting potential. Tetrodotoxin at 1 μM completely blocked the current through the BTX-modified channels.

The action of BTX can be moderately speeded up by repeated depolarizing stimuli at 1 Hz. However, the final voltage-clamp

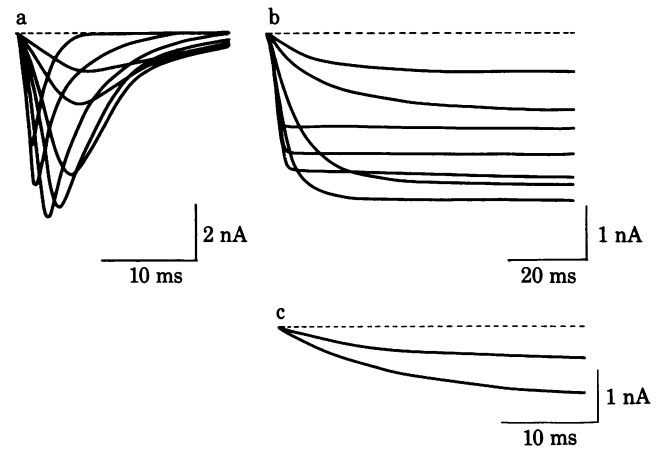


FIG. 1. Time course of Na⁺ current. (a) Normal. The membrane potential was held at -100 mV and depolarized to -45 , -40 , -35 , -30 , -20 , -5 , and $+15$ mV. (b) After saturating concentration (5 μM) of BTX was added to the external solution for 30 min. The membrane potential was held at -110 mV and was depolarized to -90 , -85 , -75 , -65 , -40 , -35 , and -25 mV. The inward Na⁺ current is no longer inactivated. Note the differences in current and time scales between *a* and *b*. (c) After saturating concentration (5 μM) of BTX was added to the external solution for 30 min. The membrane potential was held at -110 mV and was depolarized to -90 and -85 mV. Time scale same as in *a*.

currents were the same whether or not depolarizing pulses were applied.

Na Channel Activation. The properties of BTX-modified Na channels were determined for the case in which all Na channels were saturated with the toxin, as evidenced by the complete elimination of the normal component of Na⁺ current (Fig. 1*b*). The maximal amplitudes of both the normal and BTX-modified currents are plotted as a function of membrane potential in Fig. 2. These current–voltage curves show that BTX shifted the reversal potential 32 mV in the hyperpolarizing direction. The voltage shift for Na channel activation can be seen more clearly in the corresponding normalized conductance–voltage curves of Fig. 3*a*, which show that the shift is about 50 mV in the hyperpolarizing direction and that there is no significant change in shape. The mean (\pm SD) slope of the normalized conductance–voltage curve for normal sodium channels is 5.5 ± 2.0 mV per e -fold change of membrane potential (from six cells); for BTX-modified channels, the slope is 5.5 ± 1.8 mV (from eight cells).

The rate of Na activation is considerably slower in BTX-treated cells (Fig. 1). Also, in contrast to the sigmoidal time

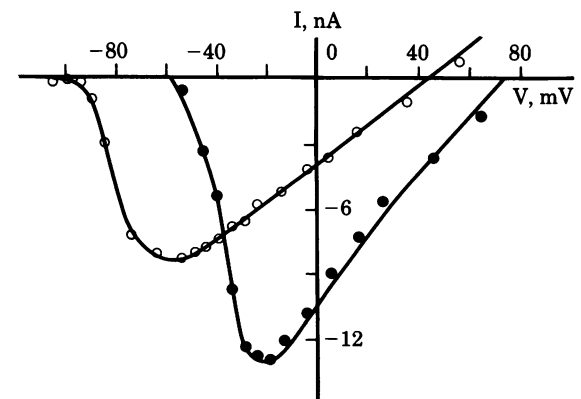


FIG. 2. Current–voltage relationship of inward Na⁺ current for normal (●) and BTX-modified (○) Na channels. The solid line is drawn by eye. Note that BTX-modified Na⁺ current activates at a potential more hyperpolarized than that for the normal Na channels.

course of the normal Na^+ current, the BTX-activated Na^+ current follows first-order kinetics (as can be seen at the faster time scale in Fig. 1c). Activation time constants are plotted as a function of membrane potential for both types of channels in Fig. 3b. For normal channels, the time constants are based on Hodgkin-Huxley formulation (17, 18); for BTX-modified channels, the time constants are based on first-order kinetics. Fig. 3b indicates that BTX shifts the activation time constant about 50 mV in the hyperpolarizing direction and also increases the magnitude of the maximal time constant about 6-fold.

Na Fast and Slow Inactivation. In control neuroblastoma cells, the Na^+ current inactivates when the membrane potential is depolarized. We determined the value of the steady-state inactivation parameter h_∞ by measuring the relative peak Na^+ current during -20 -mV test pulses after 100-ms prepulses of various amplitudes (Fig. 4 Left). The potential where $h_\infty = 0.5$ was about -70 mV. The time constant of inactivation τ_h (not shown) ranged from 6 to 27 ms between potentials of -110 mV and -40 mV, and its peak occurred at -70 mV. BTX completely abolished this fast inactivation of Na^+ current at all potentials.

The normal Na^+ current in NG108-15 also exhibited inactivation in the range of seconds. This slow inactivation (S_∞) was measured by holding the membrane potential at various depolarizations for 40–50 sec. The peak Na^+ current was then measured during -20 -mV test pulses. Before each test pulse, the membrane potential was returned to the holding value for an interval that was long compared to fast inactivation but short compared to slow inactivation, in order to eliminate fast inactivation. (For details of this measurement, see *Inset* and legend of Fig. 4.) The relative peak Na^+ current showed a sigmoidal decrease as the membrane became more depolarized (Fig. 4 Right). The $S_\infty - V$ curve has a midpoint (where $S_\infty = 0.5$) at -43 mV. BTX completely abolished this slow inactivation of Na^+ current at all potentials. Thus, BTX completely abolishes both fast and slow inactivation of Na^+ current.

Reversibility of BTX Action. The effect of $5 \mu\text{M}$ BTX on the Na^+ current in NG108-15 neuroblastoma cells is not readily reversible. When the BTX-treated cells were washed constantly both externally and internally for up to 1 hr, the steady-state Na^+ current did not decrease substantially. The normal Na^+

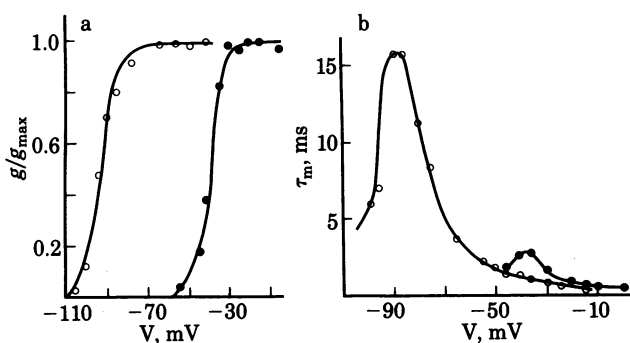


FIG. 3. Voltage dependence. (a) Relative Na conductance, g/g_{max} , before (●) and after (○) BTX ($5 \mu\text{M}$) treatment. g_{max} is maximal conductance. g is determined from $g = I_{\text{Na}}^*/(V - V_{\text{rev}})$ in which I_{Na}^* is peak Na^+ current if no inactivation exists and V_{rev} is the reversal potential of Na^+ current. The solid line is drawn according to $g/g_{\text{max}} = [1 + \exp(V_0 - V)/K]^{-P}$. Before BTX treatment, $P = 3$, $K = 5.5$ mV, $V_0 = -40$ mV, $V_{\text{rev}} = 72$ mV. After BTX treatment, $P = 1$, $K = 5.7$ mV, $V_0 = -85$ mV, $V_{\text{rev}} = 40$ mV. (b) Time constant of Na activation (τ_m) before (●) and after (○) BTX ($5 \mu\text{M}$) treatment. τ_m is determined by fitting the Na^+ current to the equation $I_{\text{Na}} = I_{\text{Na}}^*[1 - \exp(-t/\tau_m)]^P [h_\infty + (1 - h_\infty) \exp(-t/\tau_h)]$. h_∞ is the fast-inactivation parameter and τ_h is the corresponding time constant. They are obtained from experiments such as those described in Fig. 4 Left. Before BTX treatment, $P = 3$; after BTX treatment, $P = 1$. The solid lines are drawn by eye.

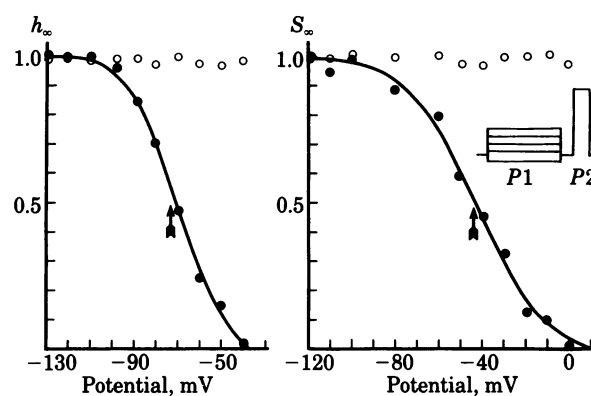


FIG. 4. Voltage dependence of steady-state Na inactivation. (Left) Fast inactivation (h_∞) before (●) and after (○) BTX ($5 \mu\text{M}$) treatment. The data points are the peak Na^+ currents obtained with the pulse sequence shown in the *Inset*. The holding potential was -100 mV for normal Na^+ current and -110 mV for BTX-modified Na^+ current. The duration of P1 was 100 ms. The potential was subsequently returned to the holding potential for 1 ms and then was stepped to a test potential of -20 mV for normal Na^+ current or of -60 mV for BTX-modified Na^+ current. The current was normalized to the peak Na^+ current ($I_{\text{Na}0}$) at the same test potential without a prepulse. The solid line is drawn according to $I_{\text{Na}}/I_{\text{Na}0} = [1 + \exp(V + 71)/10.3]^{-1}$, in which V is the potential during P1. (Right) Slow inactivation (S_∞) before (●) and after (○) BTX ($5 \mu\text{M}$) treatment. The data points are obtained as in *Left*, except that the prepulse length was 50 sec, the interval between P1 and P2 was 200 ms, and the test potential was -20 mV for normal Na^+ current and -60 mV for BTX-modified Na^+ current. The solid line is drawn according to $I_{\text{Na}}/I_{\text{Na}0} = [1 + \exp(V + 43)/14.3]^{-1}$.

current did not reappear. We do not know at present whether the effect of BTX can be reversed with longer washing time. By comparison, the action of BTX is reversible with about 90 min of washing in electric eel (19) and with about 60 min of washing in N18 neuroblastoma cells (20). BTX effects are not reversible in frog node (9, 21) or in squid (7).

DISCUSSION

Na channels in hybrid NG108-15 neuroblastoma cells are similar in gating properties to those found in squid axon (17, 18, 22, 23), frog node (24, 25), frog muscle (26), *Myxicola* (27, 28), neuroblastoma NIE115 (29, 30), and N18 (unpublished data). Electrophysiological studies of the action of BTX on Na channels have been performed in squid (6, 7), frog (9, 21), and electric eel (19). BTX depolarizes the membrane potential in NG108-15 as well as in all the above, although for electric eel this occurs only after the repetitive application of depolarizing pulses.

After addition of BTX to neuroblastoma NG108-15 cells, a new component of Na^+ current with different kinetics and different voltage dependence gradually developed. This BTX-modified Na^+ current developed concurrently with the disappearance of normal Na^+ current. Both components were completely blocked by tetrodotoxin. These observations strongly support the view (9) that BTX does not create new channels but modifies the existing Na channels. Because of the complexity of analyzing the Na^+ current when two types of channels (normal and BTX-modified channels) exist simultaneously, we have focused our analysis on the case in which all the channels are modified by BTX.

The reversal potential of normal Na channels in cells perfused with CsF is 70 – 80 mV. This is consistent with a permeability ratio $\text{Cs}^+/\text{K}^+/\text{Na}^+$ of $0.01:0.1:1.0$. In $5 \mu\text{M}$ BTX, reversal potential of the Na^+ current shifted 30 – 40 mV in the hyperpolarized direction. This is consistent with a permeability ratio of $\text{Cs}^+/\text{K}^+/\text{Na}^+$ of $0.14:0.38:1.0$, which is the ratio that was found for BTX-modified channels in frog node (21). It is also consistent with flux measurements of ions and organic compounds in neu-

roblastoma N18 cells, which show that BTX-modified Na channels are less selective than normal Na channels (31).

Our results on the effects of BTX on Na channels in NG108-15 neuroblastoma cells under voltage clamp can be summarized as follows: (i) The conductance-voltage curve is shifted about 50 mV in the hyperpolarizing direction with no change in shape. (ii) The τ_m -voltage curve is shifted about 50 mV in the hyperpolarizing direction with a 6-fold increase in the maximum value of τ_m . (iii) Activation kinetics are changed to first order. (iv) Fast inactivation is eliminated. (v) Slow inactivation is eliminated.

The first four of these effects are qualitatively similar to effects of BTX on frog node (9, 21). It is also possible to make a limited comparison of our conductance-voltage curve (Fig. 3a) with results of flux measurements. Catterall (10) performed flux experiments with BTX on N18 cells for membrane potentials between -40 mV and 0 mV. Over this range of potentials, he found that Na conductance is constant, in agreement with Fig. 3a.

In order to compare the Na conductances for BTX-modified channels (which do not inactivate) and for normal channels, it is necessary to correct the normal channels for inactivation. With this correction, in almost all the cells tested, the maximal Na conductance (\bar{g}_{Na}) of BTX-treated cells was slightly more than half that of control cells. We cannot determine how much of this difference is related to a loss of channels caused by the addition of BTX and how much is related to a difference in the single-channel conductances of the two types of channels. However, we can place a limit on the ratio of the single-channel conductances. If no channels were lost, the single-channel conductance for BTX-modified channels would be at least one-half that of normal channels. If there were channel loss, the modified channel conductance would be higher. From the single-channel conductance obtained directly on normal neuroblastoma cells, 9 pS (D. Corey and C. F. Stevens, personal communication), the BTX-modified channel conductance is not less than 4.5 pS. This is about 50% higher than the single-channel conductance, 3 pS, estimated from binding studies of [H^3]BTX and scorpion toxin (10, 32). This difference may arise from the binding of labeled toxin to nonfunctional Na channels.

The shifts of the conductance-voltage curve and the τ_m -voltage curve are consistent with a model in which the charges of BTX molecules change the local field "seen" by the channel gate. Because the BTX molecules are positively charged at physiological pH (8), the direction of the shifts is consistent with their binding to the inner surface of the membrane. Alternatively, BTX may modify the conformation of the gating molecules in such a way that the charge distribution on the molecules is altered. Similar explanations for the voltage shift have been given by Khodorov (21).

If we assume that the voltage dependence of conductance arises from transitions between closed and open states of the channel involving the motions of charged groups or dipoles across the membrane, the slope of the conductance-voltage curve gives the number of electronic charges that move across the membrane potential drop to open one channel. In our case, the slopes correspond to approximately five electronic charges for both normal and BTX-modified channels. Thus, the modification of Na channels by BTX does not involve a change in the net charge movement.

Armstrong and Bezanilla (33) have proposed that, upon depolarization, resting Na channels must pass through several closed states in order to reach the open state. According to this model, the higher-order activation kinetics for normal channels implies that several of the transitions have comparable rates. The lack of change in net charge movement along with the change to first-order kinetics and slower activation suggests that BTX does not change the states themselves but rather slows

down one of the transitions, causing it to become rate-limiting.

The elimination of slow inactivation by BTX is a new result. Previous studies have shown that slow inactivation is not eliminated by drugs that eliminate fast inactivation, such as Pronase (28) and *N*-bromoacetamide (34). The fact that BTX induces changes in channel properties as diverse as selectivity, activation, fast inactivation, and slow inactivation, even though the BTX molecule binds to a single site, suggests that the site is located at a part of the channel that is crucial to its function.

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