

The Properties of Batrachotoxin-modified Cardiac Na Channels, Including State-dependent Block by Tetrodotoxin

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ABSTRACT Batrachotoxin (BTX) modification and tetrodotoxin (TTX) block of BTX-modified Na channels were studied in single cardiac cells of neonatal rats using the whole-cell patch-clamp recording technique. The properties of BTX-modified Na channels in heart are qualitatively similar to those in nerve. However, quantitative differences do exist between the modified channels of these two tissues. In the heart, the shift of the conductance-voltage curve for the modified channel was less pronounced, the maximal activation rate constant, $(\tau_m)_{\max}$, of modified channels was considerably slower, and the slow inactivation of the BTX-modified cardiac Na channels was only partially abolished. TTX blocked BTX-modified mammalian cardiac Na channels and the block decreased over the potential range of -80 to -40 mV. The apparent dissociation constant of TTX changed from $0.23 \mu\text{M}$ at -50 mV to $0.69 \mu\text{M}$ at 0 mV. No further reduction of block was observed at potentials greater than -40 mV. This is the potential range over which gating from closed to open states occurred. These results were explained by assuming that TTX has a higher affinity for closed BTX-modified channels than for open modified channels. Hence, the TTX-binding rate constants are considered to be state dependent rather than voltage dependent. This differs from the voltage dependence of TTX block reported for BTX-modified Na channels from membrane vesicles incorporated into lipid bilayers and from amphibian node of Ranvier.

INTRODUCTION

Voltage-gated Na channels are responsible for the rapidly rising upstroke of the action potential in nerve and muscle (Hodgkin and Huxley, 1952). Functional characterization of Na channels has been aided considerably by the use of

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naturally occurring toxins that bind specifically to Na channels. The toxins have also been useful in the isolation of the structural proteins of Na channels (Catterall, 1980; Tanaka et al., 1983; Rosenberg et al., 1984; Tamkun et al., 1984; Hartshorne et al., 1985; Furman et al., 1986). Tetrodotoxin (TTX), isolated from the puffer fish, and saxitoxin (STX), isolated from the dinoflagellate *Gonyaulax catanella*, block Na channels exclusively (Narahashi et al., 1964; Kao, 1966; Hille, 1968). Batrachotoxin (BTX), an alkaloid toxin extracted from the Colombian frog *Phylllobates terribilis* (Albuquerque et al., 1971; Myers and Daly, 1983), causes persistent activation of Na channels (Narahashi et al., 1971; Khodorov et al., 1975; Khodorov and Revenko, 1979; Huang et al., 1982, 1984; Quandt and Narahashi, 1982; for review, see Khodorov, 1985).

Voltage-clamp studies of Na current and radiolabeled TTX- or STX-binding assays have shown that the affinities of these toxins for Na channels did not change with membrane potential. However, several groups of investigators have found that the equilibrium dissociation constants of TTX and STX for single BTX-modified Na channels incorporated into lipid bilayers increased as the membrane potential increased from -60 to $+60$ mV (French et al., 1984; Green et al., 1984; Moczydowski et al., 1984; Hartshorne et al., 1985). Rando and Strichartz (1986) reexamined the question of whether STX block of Na channels in native membranes is also voltage dependent by studying the effect of STX on both normal and BTX-modified Na channels of node of Ranvier. They did not observe voltage dependence of the STX block in normal Na channels, nor did they find evidence of voltage dependence of the STX block in BTX-modified Na channels at potentials less than -50 mV. However, the affinities of STX for BTX-modified Na channels did decrease when the membrane potentials were greater than -50 mV. Rando and Strichartz proposed that STX block of open Na channels was voltage dependent, whereas STX block of closed and inactivated Na channels was voltage independent.

Many voltage-dependent properties of Na channels in heart and nerve are similar (Beeler and Reuter, 1970; Brown et al., 1981), but kinetic differences have been reported (Brown et al., 1981; Zilberter et al., 1982; Gintant et al., 1984; Kunze et al., 1985; Patlak and Ortiz, 1985). There are pharmacological differences as well. For example, TTX blocks Na current in the heart, but unlike its effects in nerve, the TTX block is use dependent (Baer et al., 1976; Cohen et al., 1981; Follmer and Yeh, 1986). In addition, cardiac Na channels bind TTX with a much lower affinity; the apparent K_d for cardiac cells is ~ 1 μ M (Baer et al., 1976; Cohen et al., 1981), whereas the apparent K_d for mature nerve cells is 0.05–10 nM (Ritchie and Rogart, 1977). BTX is known to have effects on cardiac membrane (Hogan and Albuquerque, 1971; Honerjager and Reiter, 1977; Catterall and Coppersmith, 1981; for review, see Honerjager, 1982; Renaud et al., 1982), but a detailed analysis of its effects on cardiac Na channels has not yet been carried out.

Additional comparisons of Na channels from different tissues are of particular interest because mammalian rat brain Na channel genes seem to be a multigene family (Noda et al., 1986). Our goals in these experiments were to compare the actions of BTX on Na channels in heart and nerve, and to examine the TTX

block of BTX-activated cardiac Na channels. Preliminary results of these studies have appeared in an abstract (Huang et al., 1986).

METHODS

Cell Culture

Single cardiac cells were prepared from the ventricular muscle of neonatal rats. The cells were grown as described, with some modifications (Yatani and Brown, 1985). Briefly, ventricles were removed from 8–10 neonatal rats and placed in Ca^{++} - and Mg^{++} -free Hanks' solution. Each ventricle then was cut into 8–10 pieces and incubated in Ca^{++} - and Mg^{++} -free Hanks' solution containing 0.05% trypsin, 0.01% collagenase, and 0.01% DNAase. After 5 min incubation, the supernatant was discarded. The same incubation procedure was repeated with fresh enzyme, and the supernatants were then collected in medium. The medium solution contained 45% Dulbecco's modified Eagle's medium, 45% F12, and 10% fetal calf serum. The cells then were washed by centrifugation and resuspended in medium; they were then seeded at a density of 1×10^5 cells/cm² on a coverglass and incubated at 37°C. These cells were used within 1–2 d. The cells were spherical in shape and had diameters of 10–15 μm , and the Na currents in these cells could be controlled well during voltage clamp (Kunze et al., 1985).

The composition of external solution used was (millimolar): 60 NaCl, 5 4-aminopyridine, 5 CsCl, 10 glucose, 10 HEPES, and 80 sucrose. 20 mM tetraethylammonium was added in the bath medium to block K channels. The pH of the solution was adjusted to 7.3 with CsOH. Since the supply of BTX was limited and the binding rate of this toxin was slow, most studies of BTX-modified Na current (I_{Na}) were on the cells that had been preincubated in medium containing 130 mM KCl, 10 mM glucose, 10 mM HEPES, and an appropriate amount of BTX for 45–60 min. This length of time was needed to allow the BTX binding to reach equilibrium. Immediately before an experiment, a coverslip was removed from the preincubation medium and placed in the experimental chamber filled with external solution. Because the effects of BTX on the I_{Na} of cardiac cells were not reversible (see Results), BTX was omitted from the perfusate. The action of TTX on the BTX-modified I_{Na} was studied by perfusing various amounts of TTX externally onto the BTX-treated cells during the experiment. The pipette solution contained (millimolar): 130 Cs-aspartate, 5 ATP, 5 EGTA, 20 NaOH, and 5 HEPES, pH 7.2.

Electrophysiology and Data Analysis

An experimental chamber was mounted on a phase microscope and all the experiments were performed at room temperature (20–23°C). I_{Na} was measured under voltage-clamp conditions using the patch-clamp method. A List EPC-7 amplifier (Medical Systems Corp., Great Neck, NY) was used to record the whole-cell current. The patch electrode was fabricated according to the method of Hamill et al. (1981). The tip diameter of the electrode was 2 μm . The current recordings remained stable for 30–60 min. Data were filtered using a four-pole filter at cutoff frequencies of 1–10 kHz. The sampling rates were twice the Nyquist limits. The data were stored on a digital tape (Nicolet, Madison, WI) and analyzed later using a PDP-11/70 (Digital Equipment Co., Maynard, MA) minicomputer. Least-squares minimization was the algorithm used for the curve fitting.

Several procedures were routinely used to keep I_{Na} controlled under voltage-clamp conditions. We reduced the series resistance error by keeping the electrode resistance between 0.5 and 2 M Ω , by using low external Na experimental solution, and by employing electronic compensation for the series resistance. If I_{Na} increased sharply near the current

threshold or a notch was present in the tail current, I_{Na} was not well controlled and the cell was discarded.

RESULTS

Time Course of BTX Action on Cardiac Na Channels

Neonate myocytes had a resting potential of -80 mV. Liquid junction potentials of $<5 \pm 3$ mV were not corrected. When the membrane potential was depolarized from a holding potential of -90 or -100 mV to a potential more positive than -45 mV, I_{Na} activated rapidly and then inactivated (Fig. 1A) in the normal manner (Kunze et al., 1985). In $1 \mu\text{M}$ BTX, I_{Na} activated at much more negative potentials (-80 mV) (Fig. 1B) and no longer inactivated at these potentials. At potentials more positive than -45 mV, where I_{Na} was normally inactivated, both a transient and a steady component were present in the BTX solution. As more

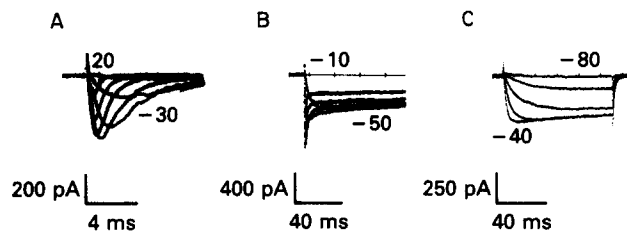


FIGURE 1. Cardiac I_{Na} 's before (A) and after (B and C) BTX treatment. Membrane potentials were stepped to different potentials in 10-mV increments. (A) Normal Na currents. The holding potential is -90 mV. (B) Na currents measured when only half of the channels were bound to BTX. This cell was incubated in $1 \mu\text{M}$ BTX for 2 h before the start of the experiment. The membrane potential was held at -100 mV. Part of the inward Na currents were no longer inactivated. (C) BTX-modified Na currents. $8 \mu\text{M}$ BTX was added to the cell. Most of the channels in this cell were converted into modified channels by BTX. Note the difference in time scale between A and B and between A and C.

BTX molecules bound to Na channels, the transient I_{Na} gradually disappeared and, at the same time, the steady inward current gradually increased. These observations confirm that the kinetic properties of the Na channels bound by BTX are different from those of normal channels. 30–40 min later, BTX binding reached equilibrium and the waveform of I_{Na} remained constant. The peak transient current in this case was the sum of the currents flowing through both normal and BTX-modified Na channels (Fig. 1B). Since the normal I_{Na} completely inactivated after 20 ms, the steady state current, measured at the end of a long (0.1–2 s) pulse, flowed through BTX-modified channels. In order to avoid ambiguities that might arise in resolving current records of mixtures of normal and BTX-modified I_{Na} 's, we determined the properties of BTX-modified Na channels only in cells in which 95–100% of the Na channels were modified. An example of such currents is shown in Fig. 1C. The effect of BTX could not be readily reversed in myocytes. After the BTX-treated cells were washed continu-

ously with a toxin-free external solution for >1 h, the BTX-modified I_{Na} did not decrease, and the normal I_{Na} did not reappear.

The peak and/or steady state currents are plotted as functions of membrane potential in Fig. 2. The normal I_{Na} began to activate at -45 mV (Fig. 2A), and the BTX-modified I_{Na} became noticeable at -80 mV (Fig. 2C). The current-voltage (I - V) curve of BTX-modified Na channels was shifted to the left. The reversal potential for the modified I_{Na} also was shifted ~ 10 – 15 mV in the hyperpolarizing direction. In cells that had a fraction of Na channels modified by BTX, the I - V curve showed two minima (Fig. 2B). The I - V relationship of the peak currents was similar to that of normal Na channels, and the I - V relationship of the steady state current was similar to that of BTX-modified Na channels.

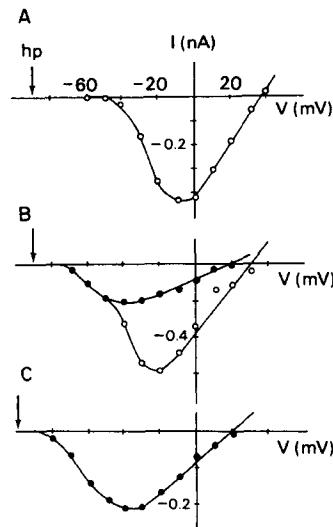


FIGURE 2. I - V relationship of peak (\circ) and steady state (\bullet) (measured at $t = 100$ ms) I_{Na} . (A) Control. (B) $1 \mu\text{M}$ BTX was applied to the cells. About 50% of the Na channels were modified by BTX in this cell. (C) $8 \mu\text{M}$ BTX was added to the cells. 95% of the Na channels were modified by the toxin in these cells. The I - V curve in B has two minima. BTX-modified Na currents activate at potentials more hyperpolarized than normal.

Activation and Inactivation Properties of BTX-activated Na Channels

In order to analyze the voltage shift for the activation of modified channels, the conductance (g) of both normal and modified channels was calculated according to $g = I_{Na}^{\#} / (V - V_{rev})$. $I_{Na}^{\#}$ is the I_{Na} when an inactivation process is not present. V_{rev} is the reversal potential of this current. The conductance vs. voltage curves for normal and BTX-modified Na channels are shown in Fig. 3. The curve for modified channels was shifted 30 mV in the hyperpolarizing direction. The slope of the conductance voltage curves was 7.8 and 8.3 mV per e-fold change of membrane potential for normal Na channels and for BTX-modified Na channels, respectively. This corresponds to the movement of 3–4 equivalent gating charges across the entire membrane field for the opening of a normal Na channel or a BTX-modified Na channel. Thus, the net charge movement remained the same after BTX treatment.

The activation time constant, τ_m , of the normal I_{Na} was determined by fitting the I_{Na} to the equation $I_{Na} = I_{Na}^{\#} [1 - \exp(-t/\tau_m)]^3 \cdot [h_{\infty} + (1 - h_{\infty})\exp(-t/\tau_h)]$

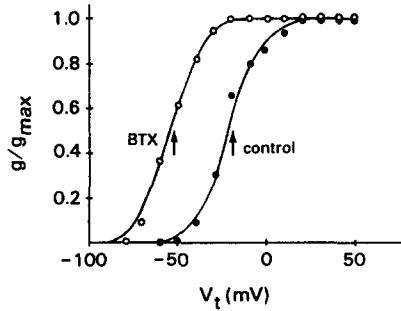


FIGURE 3. Voltage dependence of relative cardiac Na conductance (g/g_{max}) before (●) and after (○) BTX ($8 \mu M$) treatment. g was determined from the equation $g = I_{Na}^* (V - V_{rev})$, in which I_{Na}^* was the peak I_{Na} if no inactivation existed as described in the text and the legend to Fig. 4. The solid lines were drawn according to $g/g_{max} = \{[1 + \exp(V_o - V)]/k'\}^{-p}$. For normal I_{Na} (●), the least-squares fit for k' was 7.8 mV, for $V_o = -23.3$ mV, and for $g_{max} = 10.5 \times 10^{-8}$ mho when p was set at 3 and V_{rev} was 30 mV. For steady state I_{Na} (○), $k' = 8.3$, $V_o = -55$ mV, and $g_{max} = 3.5 \times 10^{-8}$ mho when p was set at 1 and V_{rev} was set at 20 mV.

(Oxford, 1981), where h_{∞} is the fast inactivation parameter, and τ_h is the corresponding time constant. After BTX was applied, I_{Na} no longer inactivated and the activation of I_{Na} followed first-order kinetics. The τ_m for the BTX-modified I_{Na} could be obtained easily using the equation $I_{Na} = I_{Na}^* [1 - \exp(-t/\tau_m)]$. I_{Na}^* here is simply the steady state level of I_{Na} . The voltage dependences of τ_m for both normal and modified Na channels are shown in Fig. 4. The τ_m values for BTX-modified channels ranged from 1 to 20 ms, whereas the τ_m values for normal Na channels were between 0.5 and 0.1 ms. Over most of the range of potentials, the activation rates of the BTX-modified I_{Na} were considerably slower than those of normal Na channels, and the τ_m - V curve of the modified I_{Na} was also shifted in the hyperpolarizing direction. Normal Na channels inactivated completely during prolonged depolarization.

The steady state inactivation parameter h_{∞} was determined by measuring the relative peak I_{Na} at a -10 -mV test pulse after holding the membrane potential at various levels for 100 ms (Fig. 5A). The h_{∞} - V curve was fitted with a Boltzmann equation: $I_{Na}/I_{Na}^o = [1/\exp(V - V_o)/k']^{-1}$. I_{Na}^o is peak I_{Na} at the test potential

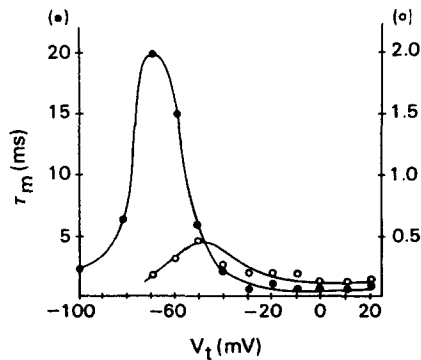


FIGURE 4. Voltage dependence of time constant of activation of cardiac I_{Na} (τ_m) before (○) and after (●) treatment with BTX ($8 \mu M$). τ_m is obtained by fitting I_{Na} to the equation $I_{Na} = I_{Na}^* [1 - \exp(-t/\tau_m)] [h_{\infty} + (1 - h_{\infty}) \exp(-t/\tau_h)]$. h_{∞} is the steady state value for the fast component of inactivation; τ_h is the time constant of fast inactivation. For the normal Na current (○), p is set at 3. For BTX-modified I_{Na} 's (●), $p = 1$, $h_{\infty} = 1$. Note the difference in the τ_m scale between two curves.

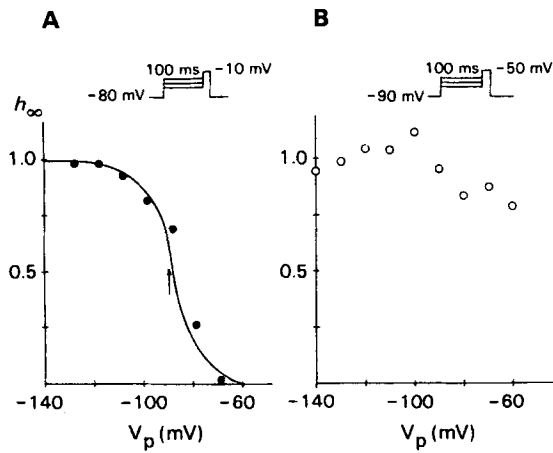


FIGURE 5. Voltage dependence of I_{Na} fast inactivation (h_{∞}) (A) before (●) (B) after (○) BTX ($8 \mu M$) treatment. The data points were the peak I_{Na} obtained at the test potential. The pulse protocols are illustrated in the inset. The current was normalized to the peak I_{Na} at the same test potential without a prepulse. The solid line was drawn according to $I_{Na}/I_{Na}^0 = [1 + \exp(V + 88)/5.5]^{-1}$. BTX reduced the fast inactivation by 85–90%.

without a prepulse, V is the prepulse potential, V_o is the potential at which $I_{Na}/I_{Na}^0 = 0.5$, and k' is the slope of the curve. In this case, V_o was about -80 mV. BTX abolished almost all (90%) of the fast inactivation of I_{Na} at all membrane potentials (Fig. 5B).

The normal cardiac I_{Na} also decayed with another time constant in the range of seconds (Fig. 6). The steady state slow inactivation parameter S_{∞} was obtained

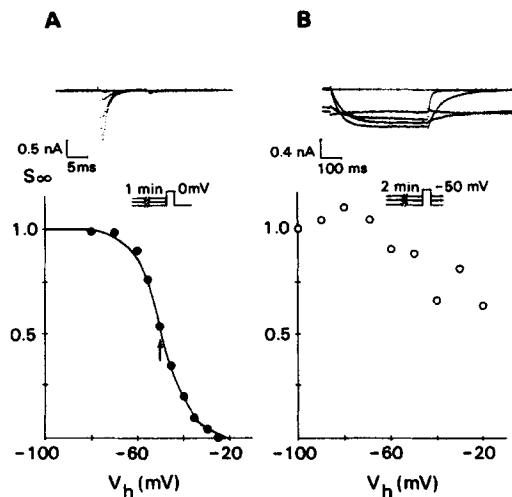


FIGURE 6. Voltage dependence of I_{Na} slow inactivation (S_{∞}) of (A) normal Na channels (●) and (B) BTX-modified Na channels (○). The current records are given at the top of each curve. The protocols used are shown in the inset. (A) The normal peak I_{Na} shown here was measured at 0 mV after holding the membrane potentials at -80 , -70 , -60 , -50 , -40 , and -30 mV for 1 min. The current was normalized to the peak I_{Na} measured as the membrane potential was held at -80 mV. The solid line was drawn according to $I_{Na}/I_{Na}^0 = [1 + \exp(V + 50)/6.3]^{-1}$. (B) The BTX-modified currents were measured at -50 mV when the membrane potential was held at -100 , -80 , -60 , and -40 mV for 2 min. BTX reduced the slow inactivation by ~ 75 – 70 %.

by measuring the relative peak I_{Na} at -50 mV (for BTX-modified channels) or 0 mV (for normal Na channels) after membrane potentials were held at various prepulse levels for 1–2 min. The voltage dependence of S_{∞} is given in Fig. 6. The curve was fitted with the same equation as described for h_{∞} , except that $V_o = 50$ mV and $k' = 6.3$ in this case (Fig. 6A). 75–80% of slow inactivation was eliminated with the addition of BTX (Fig. 6B).

Voltage-dependent TTX Block of BTX-modified Na Channels

As in the case of normal cardiac Na channels, BTX-modified channels were blocked by externally applied TTX. Fig. 7 illustrates the inhibition of the BTX-modified I_{Na} by $0.3 \mu\text{M}$ TTX at two different membrane potentials. TTX ($0.3 \mu\text{M}$) blocked 56% of the modified I_{Na} at -50 mV, but only 30% at -10 mV (Fig.

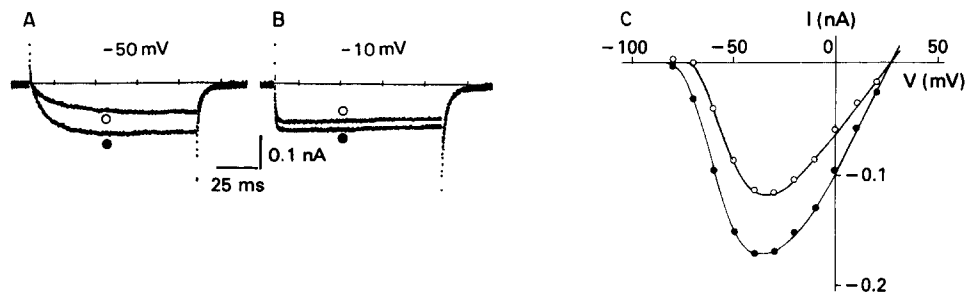


FIGURE 7. Effect of TTX on current (A and B) and I - V relationship of BTX-modified I_{Na} (C). (A) I_{Na} measured at -50 mV before (●) and after (○) the addition of $0.3 \mu\text{M}$ TTX. (B) I_{Na} obtained as in A except the test potential was -10 mV. Note the difference in the extent of block between A and B. (C) The voltage dependence of BTX-modified I_{Na} with and without $0.3 \mu\text{M}$ TTX clearly showed a decreasing TTX block as the membrane potentials became more depolarized.

7, A and B). Hence, the block of BTX-modified channels by TTX appeared to be voltage dependent, with a larger fraction of I_{Na} blocked at more hyperpolarizing potentials (Fig. 7, A and B). The voltage dependence of the BTX-modified I_{Na} , with and without $0.3 \mu\text{M}$ TTX, is illustrated in Fig. 7C. TTX reduced the BTX-modified I_{Na} by 98% at -80 mV, by 58% at -60 mV, and by only 35% at -30 mV. The steady state Na conductance was calculated from I_{Na} 's measured at different membrane potentials using the equation described in the previous section. The normalized conductance was plotted as a function of potential before and after TTX treatment (Fig. 8). The g - V curve in the presence of TTX was shifted by 7 ± 2 mV (six cells) to more positive potentials. The region of potentials where the voltage-dependent TTX block occurred could be clearly illustrated by plotting the normalized modified I_{Na} , measured in the presence and in the absence of TTX, as a function of potential. Fig. 9 shows that the TTX block was reduced between -80 and -40 mV. No further reduction of block was observed between -40 and $+50$ mV (Fig. 9).

If the TTX-binding time constant to BTX-modified channels in cardiac cells were on the order of seconds, as was observed in lipid bilayer-incorporated

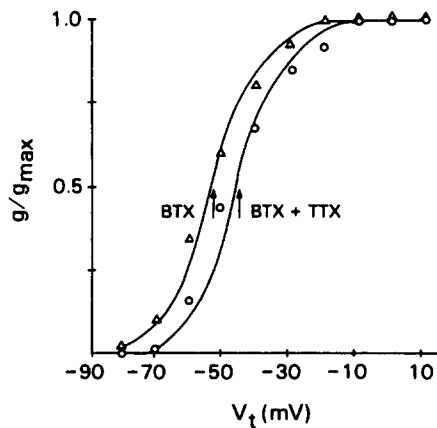


FIGURE 8. Voltage dependence of relative Na conductance for BTX-modified Na channels before (Δ) and after (\circ) adding $0.3 \mu\text{M}$ TTX to the cells. The solid lines were drawn using the same method described in Fig. 3. $V_o = -55 \text{ mV}$ and $g_{\text{max}} = 3.77 \times 10^{-8} \text{ mho}$ for BTX-modified cardiac Na current and $V_o = -48 \text{ mV}$ and $g_{\text{max}} = 3.11 \times 10^{-8} \text{ mho}$ after TTX treatment. $V_{\text{rev}} = 25 \text{ mV}$ and $k' = 7.0$ for both curves.

membrane vesicles or in node of Ranvier (French et al., 1984; Rando and Strichartz, 1986), the depolarizing pulses of 0.1 s duration used in our experiments may not have been long enough to allow TTX to reach equilibrium at each test potential. To ensure that this was not the case, we depolarized the membrane potential to different levels for 1 s and determined the TTX block by measuring the modified I_{Na} at the end of the depolarizing pulse (Fig. 10A). This protocol gave results similar to those shown in Fig. 7. The level of block remained constant after 0.1 s. No relaxation phenomena, owing to slow TTX binding kinetics, were observed (Fig. 10A). We checked this point further by using a repetitive pulsing protocol. The cells were stimulated at 10 Hz with 80-ms depolarizations for 10 s (Fig. 10B). We assumed that the interval between pulses (20 ms) was too short to allow the reequilibration of TTX to the holding potential. The toxin would then reach its new steady state after the application of a large number of pulses. Repetitive pulses did not result in any substantial

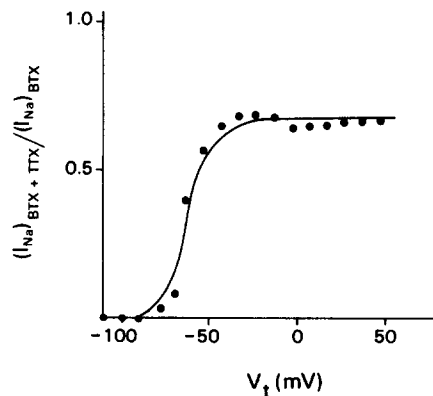


FIGURE 9. The extent of TTX ($0.3 \mu\text{M}$) block as a function of test potentials. The steady state I_{Na} , obtained from an experiment similar to that in Fig. 7, was normalized to the modified I_{Na} measured in the absence of TTX (I_{Na}^o). The solid line was drawn according to $I_{\text{Na}}/I_{\text{Na}}^o = [1 + \exp(60 - V)/7.4]$. TTX block was limited to the region of potentials where closed to open gating of the modified channels occurred.

change of current magnitude (Fig. 10B). The level of TTX block remained constant after, at most, the third pulse at all test potentials (Fig. 10B). Moreover, this is a worst estimate since we cannot exclude a contribution from inactivation of the BTX-modified channels (see Figs. 5 and 6). Thus, unlike experiments on the bilayer-membrane vesicle preparation or frog node, the TTX binding of BTX-modified channels was much faster in rat neonatal cardiac cells.

The affinities of TTX for its receptor were determined at -50 and 0 mV by measuring the modified I_{Na} at the end of the 0.1 -s depolarizing pulse (Fig. 11). The error bars represent the experimental variations among data from eight cells. The data were fitted by a simple 1:1 reaction scheme between TTX and its receptor site. The solid lines corresponded to a modified Michaelis-Menten equation, $[T]/(K_{app} + [T])$, where $[T]$ is the concentration of TTX and K_{app} is

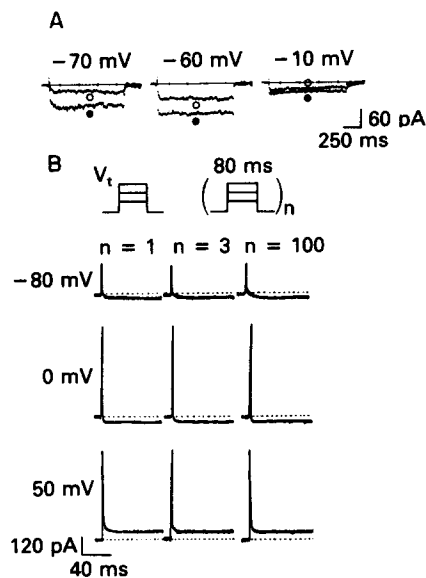


FIGURE 10. The effect of sustained (A) and repetitive (B) pulses on the block of BTX-modified I_{Na} by TTX. (A) I_{Na} measured at indicated potentials before (●) and after (○) the addition of $0.3 \mu\text{M}$ TTX. Note the difference in the extent of block at different potentials. No change in the level of block owing to relaxation of TTX binding was observed in these records. (B) The protocols used in the repetitive pulse were experiments described in the text (top); the membrane currents were displayed on a chart recorder (bottom). The modified I_{Na} was measured at the indicated potential when $n = 1, 3$, and 100 . The block remained constant after, at most, the third pulse.

the apparent dissociation constant of TTX. The apparent dissociation constants, K_{app} , were $0.23 \mu\text{M}$ at -50 mV and $0.69 \mu\text{M}$ at 0 mV. TTX was about three times more potent in blocking BTX-modified Na channels at -50 than at 0 mV. For a normal I_{Na} studied under comparable conditions, the K_{app} was $\sim 0.5 \mu\text{M}$.

Use-dependent Block of TTX

TTX block of the normal I_{Na} in cardiac cells was enhanced by repetitive depolarization (Cohen et al., 1981). Use-dependent block of TTX was also observed in our preparation. With the addition of $0.3 \mu\text{M}$ TTX, the normal I_{Na} elicited by the first pulse in a pulse train was reduced to 63% of the control value. As repeated pulses were applied to the cells, the normal I_{Na} progressively decreased. After five to six pulses, the normal I_{Na} reached a new steady state (43% of control). The time constant for the development of this use-dependent block was 1.4 s.

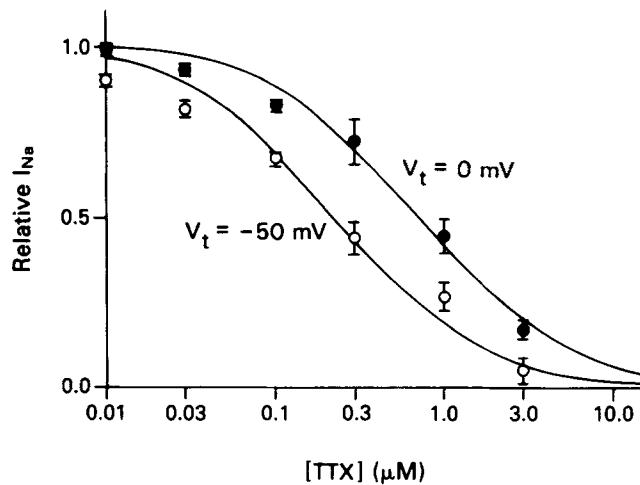


FIGURE 11. Dose-response curve for TTX block of BTX-modified cardiac Na channels at two test potentials. Cells were held at -100 mV. Various concentrations of TTX were perfused into the bath. After 3–4 min, BTX-modified Na currents were measured at $t = 100$ ms using a test pulse to -50 (○) or 0 (●) mV. The data were collected from eight cells. The error bars represent standard deviations.

In BTX-treated cells, $0.3 \mu\text{M}$ TTX decreased the modified I_{Na} to 43% of control in the first pulse. Additional pulsing did not enhance the degree of TTX block. Thus, use-dependent block of TTX disappears almost completely in BTX-modified Na channels. Since the normal I_{Na} and the BTX-modified I_{Na} illustrated in Fig. 12 were obtained from two different cells, the fact that the level of tonic TTX block of the modified I_{Na} coincided with the final level of use-dependent TTX block of the normal I_{Na} was fortuitous. Depending on the test potential used, the level of tonic TTX block of BTX-modified channels was usually 10–15% greater than that of normal Na channels.

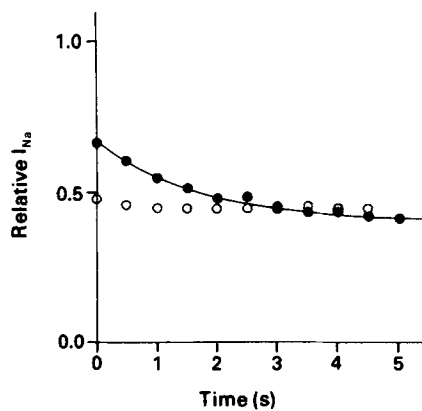


FIGURE 12. Use-dependent block of TTX of normal (●) and BTX-modified (○) cardiac Na channels. The current at $t = 0$ represents the tonic block. The cell was held at -100 mV. Test pulses of -30 mV and 100 ms duration were applied once every 0.5 s to the membrane. In the presence of BTX, the use-dependent effect of TTX disappears. The fact that the final values for normal and for modified currents were the same was coincidental.

DISCUSSION

Comparison of BTX Effects in Heart and in Nerve

The actions of BTX on Na channels have been studied in a variety of nerve cells, such as squid axon (Narahashi et al., 1971), frog node of Ranvier (Khodorov et al., 1975; Khodorov and Revenko, 1979), electric eel electroplax (Bartels-Bernal et al., 1977), and neuroblastoma cells (Huang et al., 1982, 1984; Quandt and Narahashi, 1982). In cardiac cells, as in nerve cells, BTX changes the gating the kinetic properties of I_{Na} dramatically. After application of BTX, a modified I_{Na} develops as the normal I_{Na} disappears. Both normal and modified Na channels have similar affinities for TTX. These results support the idea that BTX modifies the existing cardiac Na channels rather than creating new ones (Khodorov et al., 1975; Huang et al., 1982).

With respect to toxins, a unique property of the cardiac Na channel is that it is relatively insensitive to TTX. The apparent dissociation constant, K_{app} , for TTX block of the normal cardiac I_{Na} is $0.5 \mu\text{M}$. This value is ~ 25 – 50 -fold greater than the K_{app} for TTX block of nerve I_{Na} . Thus, a portion of the cardiac Na channels that binds TTX molecules is modified in cardiac tissue. As a result, the first question we asked was whether BTX-binding sites in heart are significantly different from those in nerve.

Table I compares selected properties of BTX-modified Na channels in several different preparations. Our results show that for these parameters, modified Na channels in cardiac cells and nerve cells behave similarly. BTX-modified Na channels turn on at much more hyperpolarized potentials (-80 to -90 mV), activate more slowly, with first-order kinetics, and do not inactivate in both tissues. The reversal potential of normal Na channels in cardiac cells is 40 mV (Fig. 2); the permeability ratio (P_{Cs}/P_{Na}), according to the constant field equation, is 0.01 . In $8 \mu\text{M}$ BTX, the reversal potential shifts 10 mV in the hyperpolarized direction. This corresponds to a permeability ratio of $P_{Cs}/P_{Na} = 0.1$. Thus, Cs^+ is more permeant in BTX-modified cardiac Na channels than in normal Na channels. This observation is consistent with results obtained from frog node and neuroblastoma cells (Khodorov and Revenko, 1979; Huang et al., 1979). Quantitative differences do exist between BTX-modified Na channels in heart and in nerve. The 30 -mV voltage shift of the conductance-voltage curve in heart is not as pronounced as the 50 – 60 -mV shift observed in nerve cells. In heart, the maximal activation rate constant, τ_m , of modified channels is 40 -fold longer than the maximal τ_m of normal Na channels. In nerve, maximal τ_m increases only ~ 6 – 10 -fold after the addition of BTX (Table I).

Most BTX-modified Na channels studied so far do not inactivate (Khodorov et al., 1975; Huang et al., 1982; review by Khodorov, 1985). However, a moderate ($<30\%$) decrease of the modified I_{Na} in response to a sustained depolarization (-100 to -70 mV) has been observed in frog node (Dubois and Colombe, 1984) and N18 cells (Zubov et al., 1983). This partial inactivation decreases at more positive test potentials and finally disappears at potentials >0 mV. This phenomenon has been interpreted as ion accumulation or depletion in the perinodal space of frog node (Dubois and Colombe, 1984), or as the

transitions of channel states from an inactivated state to a second open state (Zubov et al., 1983). The elimination of fast inactivation in modified cardiac channels is quite complete (90–95%). We have occasionally observed a 10–20% partial inactivation of the modified cardiac I_{Na} when the membrane potential was depolarized beyond -40 mV. Since the modified I_{Na} in most of our cardiac cells is devoid of fast inactivation between -40 and $+20$ mV, this partial inactivation of the modified I_{Na} in some of the BTX-treated cells is probably caused by incomplete modification of Na channels.

We did observe a moderate degree of slow inactivation, S_{∞} (Fig. 6), in the modified I_{Na} . This is different from the effect of S_{∞} measured in neuroblastoma cells (Huang et al., 1982), in which BTX completely abolishes slow inactivation.

TABLE I
Comparison of Properties of BTX-modified Na Channel Effect of BTX

| Property | Rat neonatal cardiac cell* | Neuroblastoma NG108-15 [‡] | Frog node [§] |
|--|----------------------------|-------------------------------------|------------------------|
| $K_{0.5}$ for BTX | 0.8–1.0 μ M | 0.7 μ M | 1 μ M |
| Voltage shift of g - V curve | -30 mV | -50 mV | -60 to -65 mV |
| Apparent gating charge | 3–4 | 4–5 | 4–5 |
| $(\tau_m)_{max}$ | 20.0 ms, 22°C | 16.5 ms, 22°C | 0.95 ms, 19°C |
| $(\tau_m)_{max,BTX}/(\tau_m)_{max,normal}$ | 40 | 6–7 | 10 |
| Voltage shift of τ_m curve | -30 mV | -50 mV | -60 mV |
| Decrease in h_{∞} | 90% | 95–100% | 75% |
| Decrease in S_{∞} | 75% | 95–100% | — |
| $(P_{Ca}/P_{Na})_{BTX}^{\dagger}$ | 0.1 | 0.14 | 0.15 |
| $(P_{Ca}/P_{Na})_{normal}$ | 0.01 | 0.01 | <0.013 |
| $K_{0.5}$ for TTX | 230–690 nM | 10 nM | 3.6 nM |

* From this article.

[‡] From Huang et al. (1982).

[§] From Khodorov (1985).

[†] P_{Ca} : permeability of Ca^{2+} . P_{Na} : permeability of Na^{+} .

The mechanism underlying this partial decrease of slow inactivation remains to be established.

Use-dependent TTX Block Disappears in the Presence of BTX

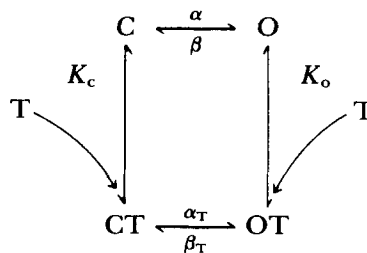
As mentioned in the previous section, Na channels in heart have a low affinity for TTX. In addition, the extent of TTX block depends on the frequency of depolarization. In Purkinje fibers and isolated adult myocytes, TTX block is markedly enhanced by repetitive suprathreshold depolarization (Baer et al., 1976; Cohen et al., 1981; Brown et al., 1982). This use-dependent TTX block was also observed in our neonatal myocytes (Fig. 12), and, as for Purkinje fibers, the binding affinity of TTX for normal Na channels was independent of voltage (Colatsky and Gadsby, 1980; Cohen et al., 1981). In the presence of BTX, the use-dependent effect of TTX disappears (Fig. 12). Our results can be explained

using the model of Cohen et al. (1981), who suggested that use-dependent TTX block of I_{Na} occurred because TTX-bound, inactivated Na channels reprimed considerably slower than toxin-free, inactivated channels. During short intervals of repolarization within a pulse train, normally inactivated channels return to the resting state, while toxin-bound channels remain inactivated. Repeated depolarizations result in the accumulation of toxin-bound channels and the extra block of I_{Na} . Since BTX-modified Na channels do not inactivate, the disappearance of the use-dependent effect of TTX in BTX-modified cells is consistent with the idea that the slow unbinding of TTX from inactivated channels is the major cause of the extra TTX block of normal Na channels.

The Voltage-dependent TTX Block of BTX-modified Na Channels Is Different in Heart and Nerve

We observed that TTX block of BTX-modified Na channels in heart was voltage dependent between -80 and -40 mV; the extent of the block was reduced at more depolarized potentials. No further reduction of the TTX block was observed at potentials from 0 to $+60$ mV. The apparent dissociation constant for TTX was $0.23 \mu\text{M}$ at -50 mV and $0.69 \mu\text{M}$ at 0 mV.

The kinetic scheme we used to interpret the voltage-dependent TTX block in the heart is given below:



where C and O represent the closed and open modified channels, respectively, and T represents TTX. We assume that the dissociation constants of TTX, K_c and K_o , are voltage independent, and the transition rates between open and closed states (α , β , α_T , β_T) vary with potential according to Boltzmann distribution. Similar models were used before in the analyses of the use-dependent block of local anesthetics in nerve and heart (Strichartz, 1973; Courtney, 1975; Hille, 1977; Hondeghem and Katzung, 1977; Lipicky et al., 1978; Bean et al., 1983).

The dissociation constants of TTX for the closed state (K_c) and for the open state (K_o) can be obtained from the following equation (cf. Appendix):

$$\frac{1}{K_{\text{app}}} = \frac{1-f}{K_c} + \frac{f}{K_o},$$

where K_{app} is the apparent TTX dissociation constant measured at a specific potential and f is the fraction of the channels open at that potential. Using the K_{app} obtained in Fig. 11, the dissociation constant for the open state, K_o , is $0.096 \mu\text{M}$ and the dissociation constant for the closed state, K_c , is $0.69 \mu\text{M}$. Thus, TTX has a higher affinity for closed BTX-modified channels. According to the model,

tighter binding of TTX to the closed state is accompanied by a shift in the conductance-voltage curve. The extent of the shift can be estimated using the relation (cf. Appendix):

$$V_o = k' \ln\{(1 + [T]/K_c)/(1 + [T]/K_o)\},$$

where k' is the slope of the conductance-voltage curve and $[T]$ is the concentration of TTX. Using the previously obtained values for K_c and K_o , $k' = 7.0$, and $[T] = 0.3 \mu\text{M}$, the calculated V_o was 7.4 mV, which agreed with the experimental results ($V_o = 7 \text{ mV}$) given in Fig. 8. The steepness of the TTX voltage dependence also corresponds well with the slope of the conductance, namely the fraction of open channels, vs. voltage curve (Fig. 9). Our results support the view that TTX binds more tightly to closed BTX-modified Na channels.

Voltage-dependent TTX or STX block was observed in BTX-modified channels isolated from skeletal muscle and brain vesicle membranes incorporated into planar lipid bilayers (French et al., 1984; Green et al., 1984; Moczydlowski et al., 1984; Hartshorne et al., 1985). These studies showed that TTX- or STX-binding rate constants were exponential functions of voltage in the range of -60 to $+60$ mV, where BTX-modified channels were always open. Moczydlowski et al. (1984) found that differently charged toxins exhibited the same voltage-dependent block and indicated that entry of the toxin molecules into an electric field could not explain voltage-dependent TTX binding. They proposed that there were high- and low-affinity states for TTX binding to open BTX-modified channels, and that the relative ratios of these two conformational states were voltage dependent.

Rando and Strichartz (1986) found that STX inhibited the BTX-modified I_{Na} from frog node of Ranvier in a complex manner. Similar to the bilayer case, the STX block decreased with pulsing potentials greater than -70 mV. However, the STX block became voltage independent when pulsing potentials were less than -110 mV. Between -110 and -70 mV, TTX block was greatest. Rando and Strichartz suggested that the binding of STX to BTX-modified Na channels was voltage dependent above -70 mV, and the complex region between -110 and -70 mV was accounted for by a TTX affinity for resting channels that was lower than its affinity for open channels at potentials near threshold. They further assumed that voltage dependences of the STX-binding rate constants for open channels were 50 mV more positive than those measured in bilayer studies.

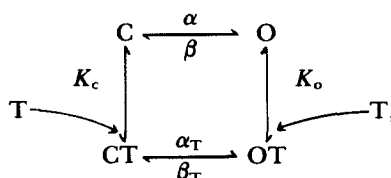
Our observations on voltage-dependent block are therefore different from those in lipid bilayers and frog node. In the heart, the binding was reduced over a relatively narrow range of potentials (-80 to -40 mV), which is the range where gating from closed to open states occurs. Thus, we interpret the changes in binding to result from the change in the closed to open conformation rather than to a voltage dependence of the binding rate constants. This is different from the frog node and the bilayer experiments, where the decrease in binding continues over potentials of -60 to $+60$ mV, at which channels are opened maximally.

We are not certain about the reasons for these different observations. One possibility is that different experimental protocols were used in these investigations. Another possibility, a more interesting one, is that the TTX-binding

proteins in the heart and in nerve or skeletal muscle are different. Low-affinity binding of TTX to cardiac Na channels is well established. Recent findings that μ -conotoxins have a high affinity for skeletal muscle Na channels, but low affinities for cardiac or nerve Na channels, further identify the existence of Na channel subtypes in various tissues (Moczydlowski et al., 1986). Whether the occurrence of tissue specificity is related to the fact that Na channels are a multigene family (Noda et al., 1986) is an intriguing question.

APPENDIX

The mathematical derivations of Eqs. 1 and 2 are given here. According to the kinetic scheme,



the dissociation constants of TTX for the closed state (K_c) and for the open state (K_o) of the BTX-modified Na channels are related to the concentration of closed ([C]) and open ([O]) modified channels, the concentration of toxin bound closed ([CT]) and open ([OT]) channels, and the concentration of tetrodotoxin ([T]) as follows:

$$K_c = \frac{[C][T]}{[CT]}$$

$$K_o = \frac{[O][T]}{[OT]}$$

At equilibrium, the ratio of [O] to [C] is

$$\frac{[O]}{[C]} = \frac{\alpha}{\beta}$$

The total concentration of channel is $R_T = [C] + [O] + [CT] + [OT]$. Substituting the values of [C], [CT], and [OT] from preceding equations, the concentration of open modified channels

$$[O] = \left(\frac{\beta}{\alpha} + 1 + \frac{[T]}{K_c} \cdot \frac{\beta}{\alpha} \cdot \frac{[T]}{K_o} \right)^{-1} \cdot R_T \quad (A1)$$

Since the measured Na current is proportional to the concentrations of open modified channels, the ratio of modified current with TTX bound (I_{Na}) to current without TTX (I_{Na}^*) is

$$\frac{I_{Na}}{I_{Na}^*} = \frac{(\beta/\alpha + 1)}{\beta/\alpha + 1 + ([T]/K_c)(\beta/\alpha) + [T]/K_o}$$

If f represents fraction of channels that are open, namely $f = [O]/([C] + [O])$, the above equation takes the form

$$\frac{I_{Na}}{I_{Na}^*} = \frac{1}{1 + ([T]/K_c)(1 - f) + ([T]/K_o)} \quad (A2)$$

Since there is a 1:1 stoichiometric relationship between TTX and modified channels, I_{Na}/I_{Na}^* can be written as

$$\frac{I_{Na}}{I_{Na}^*} = 1 - \frac{[T]}{K_{app} + [T]} = \frac{K_{app}}{K_{app} + [T]} \quad (A3)$$

where K_{app} is the apparent TTX dissociation constant. Substituting Eq. A3 into A2, we obtain the relation

$$\frac{1}{K_{app}} = \frac{1-f}{K_c} + \frac{f}{K_o}$$

We assume that rate constants of open and closed modified channels are related by Boltzmann distribution $O/C = \alpha/\beta = \exp[-q(V - V_o)/kt]$, where V is the membrane potential, V_o is the potential for $[C] = [O]$, k is the Boltzmann constant, t is the absolute temperature, and q is the gating charge. From Eq. A1, the conductance g can be written as

$$g \propto [O] = \left\{ \exp[-q(V - V_o)/kt] \cdot \left(1 + \frac{[T]}{K_c} \right) + \left(1 + \frac{[T]}{K_o} \right) \right\}^{-1} \cdot R_T$$

The maximum conductance corresponds to $V \rightarrow \infty$; therefore,

$$\frac{g}{g_{max}} = \frac{(1 + [T]/K_o)}{\exp[-q(V - V_o)/kt](1 + [T]/K_c)(1 + [T]/K_o)} \quad (A4)$$

For $g/g_{max} = 0.5$, Eq. A4 leads to

$$V_o - V = k' \ln \left(\frac{1 + [T]/K_o}{1 + [T]/K_c} \right),$$

where $k' = kt/q$. At $V_{0.5}$ of the conductance voltage curve, $V = V_o$, and the voltage shift of the curve, ΔV , as result of the addition of TTX is then

$$\Delta V = k' \ln \left(\frac{1 + [T]/K_o}{1 + [T]/K_c} \right).$$

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