BTX Modification of Na Channels in Squid Axons

I. State Dependence of BTX Action

JOËLLE TANGUY and JAY Z. YEH

From the Laboratoire de Neurobiologie, Ecole Normale Supérieure, F-75005 Paris, France; and the Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611

ABSTRACT The state dependence of Na channel modification by batrachotoxin (BTX) was investigated in voltage-clamped and internally perfused squid giant axons before (control axons) and after the pharmacological removal of the fast inactivation by pronase, chloramine-T, or NBA (pretreated axons). In control axons, in the presence of 2–5 μM BTX, a repetitive depolarization to open the channels was required to achieve a complete BTX modification, characterized by the suppression of the fast inactivation and a simultaneous 50-mV shift of the activation voltage dependence in the hyperpolarizing direction, whereas a single long-lasting (10 min) depolarization to +50 mV could promote the modification of only a small fraction of the channels, the noninactivating ones. In pretreated axons, such a single sustained depolarization as well as the repetitive depolarization could induce a complete modification, as evidenced by a similar shift of the activation voltage dependence. Therefore, the fast inactivated channels were not modified by BTX. We compared the rate of BTX modification of the open and slow inactivated channels in control and pretreated axons using different protocols: (a) During a repetitive depolarization with either 4- or 100-ms conditioning pulses to +80 mV, all the channels were modified in the open state in control axons as well as in pretreated axons, with a similar time constant of ~1.2 s. (b) In pronase-treated axons, when all the channels were in the slow inactivated state before BTX application, BTX could modify all the channels, but at a very slow rate, with a time constant of ~9.5 min. We conclude that at the macroscopic level BTX modification can occur through two different pathways: (a) via the open state, and (b) via the slow inactivated state of the channels that lack the fast inactivation, spontaneously or pharmacologically, but at a rate ~500-fold slower than through the main open channel pathway.

INTRODUCTION

The voltage-gated Na channels are intrinsic membrane glycoproteins which, in response to a depolarizing stimulus, become transiently permeable to Na ions

Address reprint requests to Dr. Joëlle Tanguy, Department of Pharmacology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, IL 60611.
through a series of conformational changes (Hodgkin and Huxley, 1952), kinetically described as the gating states of the channels. The gating properties of the channels can be specifically altered by many neurotoxins or pharmacological agents. Conversely, the gating status of the channels can modulate the interaction between the Na channels and many drugs or toxins (state dependence of drug action), as first proposed by Hille (1977) in the modulated receptor hypothesis for local anesthetic action.

Several electrophysiological studies support the hypothesis that the alkaloid neurotoxins, such as batrachotoxin (BTX), aconitine, veratridine, and grayanotoxins, interact preferentially with the open channels (Khodorov and Revenko, 1979; Leibowitz et al., 1986, 1987; Sutro, 1986; Hille et al., 1987; Barnes and Hille, 1988). In this hypothesis, the neurotoxin molecule reacts with the Na channel when once open and converts it temporarily to a toxin-channel complex (modified channel), which exhibits altered gating and pore properties (Hille, 1984; Hille et al., 1987). The differences in the lifetimes of the modified channels, which range from a few seconds for veratridine (Leibowitz et al., 1986, 1987; Sutro, 1986; Barnes and Hille, 1988) to several hours for BTX (Khodorov and Revenko, 1979), could account for most of the differences in the use dependence, reversibility, and degree and rate of modification induced by the different liposoluble neurotoxins. However, this open channel modification hypothesis has been rigorously tested so far only for veratridine. Two recent studies demonstrate that the binding of veratridine to the open channel only is sufficient to explain the use and voltage dependence of veratridine action, and the apparent gating properties of the veratridine-modified channels, at the macroscopic level (Leibowitz et al., 1986, 1987; Sutro, 1986) as well as at the single-channel level (Barnes and Hille, 1988). Although the macroscopic and single-channel properties of the BTX-modified channels have been extensively characterized in native and artificial membranes (for reviews, see Catterall, 1980; Khodorov, 1985; Andersen et al., 1986; French et al., 1986), the pathways of BTX modification remain to be determined.

The long-lasting lifetime of the BTX-modified channels (several hours) and the relatively slow rate of modification of the channels (Khodorov, 1985) render the probability of observing the channel modification at the microscopic level prohibitively small. The macroscopic measurements are therefore more favorable for investigating the BTX modification process. The BTX modification of the channels is enhanced and accelerated by repetitive depolarizations (use-dependent action) in most native membranes (Albuquerque et al., 1971; Hogan and Albuquerque, 1971; Warnick et al., 1971; Bartels-Bernal et al., 1977; Khodorov and Revenko, 1979; Huang et al., 1982, 1987b). From their pioneering study of BTX action in the Ranvier node, Khodorov and Revenko (1979) concluded that the conditioning depolarizing pulses had to be large enough to activate the channels, and applied from a holding potential negative enough to prevent the channels from inactivating. However, the analysis was complicated by the coexistence of normal and BTX-modified channels, and the protocol used could not discriminate whether the BTX modification was prevented by the fast or the slow inactivation.

We thus investigated the pathways of BTX modification in squid giant axons, a choice preparation for two main reasons. First, the fast and slow inactivation can be
pharmacologically separated by several agents, such as pronase, N-bromoacetamide (NBA), or chloramine-T, which completely remove the fast inactivation without affecting the slow one (Oxford et al., 1978; Rudy, 1978, 1981; Oxford, 1981; Wang et al., 1985). Second, BTX can modify all the Na channels irreversibly at the time scale of experiments, abolishing the fast inactivation and shifting the voltage dependence of the activation by ~50 mV toward more negative potentials (Tanguy et al., 1984; Tanguy and Yeh, 1988b). Using different protocols, we compared the degree and rate of BTX modification of the channels in the closed, open, and inactivated states, first in axons with intact inactivation (control axons) and then after the suppression of the fast inactivation by pronase, NBA, or chloramine-T (pretreated axons).

In this paper, we demonstrate that BTX modification can occur through two different pathways: through the open state, and through the slow inactivated state of the channels that lack the fast inactivation. The rate constants of BTX modification of the open and slow inactivated states are estimated in both control and pretreated axons. The relative contribution of these two pathways to the BTX modification process is discussed.

**METHODS**

The experiments were performed on isolated giant axons from squid, *Loligo pealei*, obtained at the Marine Biological Laboratory, Woods Hole, MA. After squeezing out the axoplasm by the roller technique (Baker et al., 1961), axons were inflated with an artificial internal solution. Once mounted in the chamber, axons were continuously perfused and superfused with appropriate internal and external solutions during voltage-clamp experiments.

The compositions of the external and internal solutions were as follows. The 300 mM Na external solution (300 Na+) contained (in mM): 300 NaCl, 150 tetramethylammonium (TMA)Cl, and 50 CaCl₂. The 0 mM Na external solution (0 Na+) contained (in mM): 450 TMACl and 50 CaCl₂. The external solutions were ionicly adjusted to ~1,000 mosmol with TMACl, and were buffered to pH 7.5 with 10 mM HEPES buffer. The 50 mM Na internal solution (50 Na+) contained (in mM): 20 Na glutamate, 30 Na phosphate, 200 Cs glutamate, 50 CsF, and 400 sucrose. The 300 mM Na internal solution (300 Na+) contained (in mM): 250 Na glutamate, 50 Na phosphate, 20 NaF, and 400 sucrose. The pH of the internal solution was adjusted to 7.3, and the osmolarity was adjusted with sucrose to ~1,040 mosmol. In experiments requiring a long-lasting depolarization, the internal Na concentration was adjusted to give a reversal potential ($E_{Na}$) around +50 mV, and the membrane potential was held at $E_{Na}$ during the internal application of BTX to avoid large currents flowing during the sustained depolarization. The junction potential developed between external and internal solutions was electronically nulled at the beginning of each experiment. In the text, the solutions will be referred to as external/internal Na⁺ concentration.

The drugs used in this study were all applied internally to the axon. In all experiments with BTX, the solution was freshly prepared from a 10⁻⁴ M stock solution containing 5% (vol/vol) DMSO. The DMSO final concentration, which was <0.25%, did not have any effect on the Na currents. In some experiments the fast Na inactivation was removed by pronase, chloramine-T, or NBA. The pronase solution (0.1 mg/ml) was applied to the axon for ~15 min at 10°C (pronase-treated axon), chloramine-T at 7.5 mM was applied for ~10 min (Huang et al., 1987a), and NBA at 0.3 mM was applied for 3–5 min. All these solutions were freshly prepared immediately before internal application and washed away after the complete removal of the fast inactivation. All experiments were performed at 8–10°C.

The sources of chemicals were as follows: Pronase (from *Streptomyces griseus*) was purchased...
from Calbiochem Corp. (San Diego, CA). Chloramine-T (N-chloro-p-toluenesulfonamide sodium salt) and NBA were purchased from Sigma Chemical Co. (St. Louis, MO). BTX was a generous gift of Dr. John W. Daly of the National Institutes of Health (Bethesda, MD).

The voltage-clamp experiments were performed using the conventional axial wire electrode technique. Two sets of guard electrodes on both sides of the axon and air-gaps on both ends of the axon were used to improve the space clamp as previously described (Oxford, 1981). The voltage-clamp step was generated from a computer (PDP 11/73; Digital Equipment Corp., Marlboro, MA) and membrane currents were sampled at 20-μs intervals by a 14-bit analog-to-digital converter. The P-P/4 method (Armstrong and Bezanilla, 1974) was used with the −P/4 pulse starting from the holding potential. The membrane was held at −80 or −150 mV for the control, and at −150 mV after BTX modification. The data were analyzed off-line. To determine the time constants of the exponential time courses, the data points were fitted by a least-squares algorithm for simultaneous multi-exponential fit. Average values of parameters in the table and the text are expressed as means ± SD.

RESULTS

BTX Does Not Modify the Na Channels in the Closed Resting States

Internal application of BTX from 10 nM to 5 μM for up to 1 h did not modify the Na currents when the membrane was held at very negative potentials (−150 mV) in the absence of depolarizing pulses. As illustrated by the families of Na currents shown as an inset in Fig. 1, the peak current (I_{Na,p}) amplitude was slightly increased by BTX at all potentials, reflecting a small increase in the maximal peak conductance (~10%), which reached its steady state within the first 5 min of BTX application and remained stable. This aspect of BTX action, observed in most of the experiments, was not investigated further. The time course of the decaying phase of the Na currents and the time-to-peak values were not altered, and the channels activated in the same potential range as in the control, without any change in the steepness of the voltage dependence of the peak Na conductance increase (Fig. 1). The kinetics and steady-state properties of the Na channels were thus not modified by BTX when BTX was applied at potentials where the Na channels were in their resting closed configurations. Similar results were obtained in pronase-treated axons.

Time Course of BTX Modification Elicited by Repetitive Depolarizations

In control axons, BTX modification required repetitive stimulation by depolarizing conditioning pulses (CP) applied from a negative enough holding potential (usually −80 mV). With successive short depolarizations to +80 mV (see protocol inserted in Fig. 2 B), a slow inward current developed at very negative potentials where no current was detected in the control (E_m < −60 mV), and a progressive removal of the fast inactivation occurred at potentials where the unmodified channels undergo inactivation. This dual alteration of the Na channel gating properties was monitored at two different test potentials (TP), −70 and +30 mV, as a function of the number of CP (Fig. 2 A and B).

At −70 mV (Fig. 2 A, top) the slowly activating and noninactivating inward current developing upon repetitive stimulation reached a stable maximal value after ~3,000 CP. At +30 mV (Fig. 2 A, bottom) the Na current in the control (trace labeled 0 P) increased rapidly and then decayed (fast inactivation) following a monoeponential
time course with a time constant ($\tau_h$) of 0.85 ms, reaching a steady-state level of 17% of the peak value. This steady-state current reflects the incompleteness of the fast inactivation in squid axons (Chandler and Meves, 1970; Bezanilla and Armstrong, 1977; Shoukimas and French, 1980; Oxford and Yeh, 1985). After a long train of CP, the fast inactivation was completely removed by BTX, as evidenced by the disappearance of the decaying phase of the Na current (trace labeled 4000 P). The gradual increase of the steady-state current occurred without a significant change in the time constant $\tau_{in}$ as observed during the removal of inactivation by pronase (Armstrong et al., 1973). This result suggests that BTX suppressed the inactivation in an all-or-none manner, transforming the inactivating Na channels into noninactivating ones as described in the frog Ranvier node (Khodorov and Revenko, 1979). The removal of the fast inactivation by BTX did not significantly alter the activation time course in the normal activation potential range ($E_m > -50$ mV). All these modifications of the Na currents were irreversible at the time scale of experiments (up to 4 h), even after washing away the BTX solution. All currents were completely blocked by 1 $\mu$M TTX, indicating that these currents were flowing through the Na channels.

The shift in the activation voltage dependence toward negative potentials was systematically associated with the removal of the fast inactivation occurring at more positive potentials. However, because of the irreversibility of BTX modification, meaningful dose–response curves could not be established to determine whether these two effects of BTX occurred independently or simultaneously as a result of the interaction of BTX with a single binding site. Instead, the time courses of these two phenomena were compared as a function of the number ($n$) of CP. The shift of the activation voltage dependence was determined by measuring the increase of the

![Figure 1. BTX does not modify the closed Na channels.](image)

The peak Na conductance values ($g_{Na}$) were calculated as $g_{Na} = I_{Na}/(E_m - E_{Na})$. The data points were fitted by a Boltzmann function of the form: $g_{Na}/g_{Na,max} = [1 + \exp ((E_{1/2} - E_m)/k)]^{-1}$ (Eq. 1), where $E_{1/2}$ is the potential corresponding to 50% of the maximal conductance and $k$ is the slope factor, with $E_{1/2} = -26$ and $-25$ mV, and $k = 8.9$ and 8.8 mV, before and after BTX application, respectively. The solid line represents the theoretical curve for the control. (HP = $-150$ mV, 300 Na$^+$/50 Na$^-$).
FIGURE 2. The Na channels are modified by BTX during repetitive depolarizations. In A and B, the membrane was repetitively depolarized by a train of 4-ms CP, according to the protocol inserted in B, during the internal perfusion with 3μM BTX. (A) Na current traces elicited by 10-ms depolarizing pulses (TP) to -70 mV (top) and +30 mV (bottom), before (traces labeled 0 P) and after a given number of CP (n), as indicated for each trace. In the bottom panel of A, for clarity of the figure, the peak Na currents were normalized to the peak value in the control (0 P), and the normalizing factor corresponding to each trace was also applied to the corresponding inward current elicited at -70 mV in the top panel. (B) For a given number of CP (n), the amplitude of the steady-state inward current at -70 mV and the increase of the noninactivating current at +30 mV were normalized to their respective maximum values and plotted as a function of n. The increase of the noninactivating current at +30 mV was calculated as \([I_{\text{Na,n}}(n) - I_{\text{Na,0}}(0)]/I_{\text{Na,0}}(n)\), with \(I_{\text{Na,0}}(0)\) corresponding to the steady-state current without CP, and \(I_{\text{Na,n}}(n)\) and \(I_{\text{Na,0}}(n)\) corresponding to the peak and steady-state currents after n CP, respectively. The time course of BTX modification at both -70 mV (filled triangles) and +30 mV (open squares) could be fitted by a single exponential function of n (solid line), with a constant equivalent to \(\tau(n) = 455\) pulses. (C) I-V relationships for the peak Na current in the control and the steady-state Na current after complete BTX modification by 2 μM BTX using a train of 3,200 CP (same protocol as in B). (D) The conductance values before and after BTX treatment were calculated from the \(I_{\text{Na,V}}\) in C, corrected for Ca block, and normalized to their respective maximum value. The solid lines were drawn according to the equation: \(g_{\text{Na}}/g_{\text{Na,\text{max}}} = A/[1 + \exp [(E_i - E_m)/k_1]] + (1 - A)/[1 + \exp [(E_i - E_m)/k_2]]\), with \(A = 0.01\) and 0.04, \(E_i = -100\) and -170 mV, \(E_m = -25\) and -75 mV, \(k_1 = 12\) and 25 mV, and \(k_2 = 6\) and 12 mV in the control and after BTX treatment, respectively. (HP = -150 mV, 300 Na+/300 Na-).
steady-state current at \(-70\) mV. The degree of removal of the fast inactivation was estimated at \(+30\) mV as the increase of the noninactivating component because of the relatively high steady-state level observed in the control in high internal Na\(^+\) concentration media (Oxford and Yeh, 1985). Both the amplitude of the steady-state Na current at \(-70\) mV and the increase of the noninactivating component at \(+30\) mV followed a very similar monoeponential time course, with a time constant equivalent to \(\tau(n) = 455\) pulses (Fig. 2 B). These data suggest first, that the modification of both the activation and inactivation mechanisms results from the interaction of BTX with a single receptor site, and second, that BTX modification is a slow process, a small fraction of Na channels being modified during each pulse (<0.1%; see calculations in Discussion), as previously proposed by Khodorov and Revenko (1979) in the frog Ranvier node.

A complete BTX modification, characterized by the suppression of the fast Na inactivation and a maximal shift of the activation voltage dependence in the hyperpolarizing direction, was reached after >2,000 CP with 2–5 \(\mu\)M BTX. The current–voltage (\(I_{\text{Na}}-V\)) relationships (Fig. 2 C) showed a very large increase in the Na currents at negative potentials (\(E_m < -20\) mV) after BTX modification, without any significant alteration in the slope of the linear \(I_{\text{Na}}-V\) relationships at positive potentials, indicating that the maximal conductance was unchanged. Since at negative potentials the Ca\(^{2+}\) block becomes especially important (Stimers et al., 1985; Yamamoto et al., 1985), the voltage dependence of the activation of the conductance was compared after correction for Ca\(^{2+}\) block (Fig. 2 D). The correction factor before and after BTX treatment was determined from the instantaneous \(I-V\) curves established in the presence of 1.5 mM La\(^{3+}\) and nominal zero Ca\(^{2+}\) (Tanguy and Yeh, 1988a). After BTX modification the potential corresponding to half-maximal conductance (\(E_{1/2}\)) was shifted by \(\sim 50\) mV in the hyperpolarizing direction, and the steepness of the voltage dependence of the conductance increase was decreased about twofold (see Fig. 2 D). In pronase-treated axons the same protocol of repetitive depolarizations induced a similar shift of the activation voltage dependence toward negative potentials.

The Fast Inactivation Protects the Channels from BTX Modification

To determine whether the channels could be modified in the fast inactivated state during depolarization, we compared the degree of modification induced by a sustained depolarization (10 min) before and after the selective removal of the fast inactivation by three different agents: pronase, chloramine-T, and NBA. In these experiments BTX was internally perfused while maintaining the membrane potential to a large positive value for 10 min in the absence of stimulation. After repolarizing the membrane to \(-150\) mV for 5 min, a single \(I-V\) curve was established to determine the degree of BTX modification from the extent of shift of the activation voltage dependence.

In control axons, while a repetitive depolarization promoted a complete modification, a sustained depolarization elicited little alteration of the Na currents, as illustrated in Fig. 3, A and B. The Na current at \(-80\) mV was still relatively small (Fig. 3 A, right), as compared with the large inward current elicited by repetitive depolarization (see trace labeled 3000 P in Fig. 2 A, top). At \(+20\) mV the steady-state current
Figure 3. The fast inactivation prevents BTX modification. In control axons (A and B), Na currents in response to test pulses to -80 and +20 mV (A) were recorded in the control (left) and during the internal perfusion of 2.5 μM BTX (right). The uppermost unlabeled trace represents the zero current level (in both A and C). In the right panel, during BTX application the membrane was, in the absence of pulsing, first clamped at HP = -150 mV until BTX equilibration, and then clamped at HP = E_{m} = +45 mV for 10 min and repolarized to -150 mV for 5 min before recording the Na currents traces. (B) Conductance–voltage relationships established from the maximal amplitude of the Na currents in the control (filled circles) and during the internal application of 2.5 μM BTX under two different conditions: (a) after holding the membrane potential for 10 min without pulsing at HP = E_{m} = +45 mV (open circles), (b) after a train of 3,200 CP (pulses of 4-ms duration depolarizing the membrane to +80 mV at 10 Hz) applied from HP = E_{m} = +45 mV (open triangles). The maximum conductance was only decreased by 5% after the sustained depolarization at +45 mV, and by 9% after 3,200 CP from +45 mV. The solid line was drawn through the filled circles according to Eq. 1, with $E_{1/2} = -26$ mV and $k = 9$ mV. In pronase-treated axons (C and D), Na currents in response to test pulses to -80 mV and +20 mV (C) were recorded after washing away the pronase solution (left), and in 3 μM BTX (right) during the two first test pulses elicited after clamping the membrane at HP = +40 mV for 10 min without pulsing (same protocol as in Fig. 3 A, right). (D) Steady-state conductance–voltage relationships before and after BTX modification by the same protocol as in C (right), corrected for Ca block (same correction factors as in Fig. 2 D), and normalized to their respective maximal values. The solid lines were drawn through the data points according to Eq. 1, with $E_{1/2} = -14$ mV and $k = 8.5$ mV (open circles), and $E_{1/2} = -74$ mV and $k = 9.4$ mV (filled circles). (HP = -150 mV, 300 Na_/50 Na_).
was increased by ~20\%, indicating that only a small fraction of the Na channels had been modified by BTX (cf. Fig. 3 right and left panels of A). The activation of this small proportion of modified channels accounts for the small increase of the conductance observed from -80 to -50 mV (Fig. 3 B, open circles). However, no significant shift of the activation voltage dependence in the hyperpolarizing direction was detected. The subsequent repetitive stimulation, in the same axon, by a train of 3,200 CP (4-ms CP depolarizing the membrane to +80 mV) applied from +45 mV in the presence of BTX, also failed to promote a shift of the conductance–voltage relationships (Fig. 3 B, open triangles). However, a train of 3,200 CP applied from -80 mV in the same axon (same protocol as in Fig. 2 B) was able to elicit a complete BTX modification.

In contrast, in pretreated axons a 10-min depolarization was sufficient to induce complete BTX modification. After pronase pretreatment (Fig. 3, C and D) the decaying phase of the Na current at +20 mV was virtually abolished (Fig. 3 C, left), indicating the suppression of the fast Na inactivation. The Na current at -80 mV was negligible, indicating that the removal of the fast inactivation by itself does not change the voltage dependence of the activation process in squid axons (Armstrong et al., 1973). As illustrated in Fig. 3 C, right, a 10-min depolarization to +40 mV in the presence of BTX elicited a large inward current at -80 mV, whereas the Na current at +20 mV was decreased. The decrease of the Na current at positive potentials usually observed in these experiments might reflect the reduction of the single-channel conductance that occurs after BTX modification (Quandt and Narahashi, 1982; Correa and Bezanilla, 1988). The normalized steady-state conductance–voltage curve was shifted toward negative potentials (Fig. 3 D) to the same extent as in control axons after complete BTX modification. No further shift was induced by a train of 3,200 CP subsequently applied from -150 mV, indicating that indeed all modifiable Na channels had been modified by BTX. Analogous experiments with NBA or chloramine-T pretreatment gave similar results.

Therefore, in control axons during a sustained depolarization, the development of the fast inactivation prevents BTX modification. During repetitive depolarization, the depolarization per se does not promote BTX modification but does promote the opening of the channels that become available for BTX modification.

Comparison of the Time Course of Modification Induced by Repetitive Stimulation in Control and Pronase-treated Axons

The time course of the modification induced by repetitive stimulation was compared in control and pronase-treated axons by measuring the relative increase of the steady-state current at -70 mV as indicative of the shift of the activation voltage dependence (Fig. 4). First, a train of short (4 ms) depolarizations (see protocol in Fig. 4 A, inset) elicited a complete BTX modification slightly faster in pronase-treated axons than in control axons. Regardless of the presence of the fast inactivation, the development of BTX modification followed a monoexponential time course (Fig. 4 A). The time constants were equivalent to $\tau(n) = 462 \pm 32$ pulses ($N = 3$) in control axons, and $\tau(n) = 314 \pm 29$ pulses ($N = 2$) in pronase-treated axons.

Second, a train of 20 pulses of 100-ms CP depolarizing the membrane to +50 mV (see protocol in Fig. 4 B) promoted the modification of only ~20% of the channels in
control axons, whereas >80% of the channels were modified after the removal of the fast inactivation by pronase (Fig. 4 B). The monoexponential time course of BTX modification was fivefold faster in pronase-treated axons than in control axons, with an averaged time constant equivalent to 12 ± 4 (N = 2) and 63 ± 10 (N = 2) pulses, respectively (Fig. 4 B). All these results are consistent with the open channel modification hypothesis as discussed below (see Discussion).

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Comparison of the time course of modification induced by repetitive depolarizations in control and pronase-treated axons. In A, the data points represent the steady-state inward Na current (I_{Na}) associated with the TP to −70 mV (see protocol in inset), once normalized to the one after 3,200 CP (relative I_{Na}), in a control axon (filled triangles) and in a pronase-treated axon (open triangles) in the presence of 4 μM BTX. The data points were fitted to a single exponential function of n (solid lines) with a time constant equivalent to τ(n) = 458 pulses for the control axon and τ(n) = 334 pulses for the pronase-treated axon. In B, the protocol displayed as an inset was applied in the presence of 4 μM BTX in a control axon (circles) and a pronase-treated axon (squares). The amplitudes of the steady-state Na current elicited by the TP at −70 mV after each 100-ms CP were normalized to their respective values at maximal modification (after 3,200 4-ms CPs) and plotted as a function of the number of CP (n). The data points were fitted to a single exponential of n (solid lines), with a time constant equivalent to τ(n) = 53 pulses and 8 pulses for the control and pronase-treated axons, respectively. (HP = −150 mV, 300 Na++/50 Na−).

The degree of BTX modification induced by the 4- and 100-ms protocols in pronase-treated axons plotted in terms of cumulative durations of the conditioning pulses (Fig. 5), followed a similar monoexponential time course, with time constants of 1.3 and 0.90 s, respectively. However, when the duration of the conditioning pulses was longer than 1 s, the channels were modified at an extremely slow rate with a time constant of 24 s. Since the channels enter in the slow inactivated state during
such long depolarizations, these results suggest that BTX could also modify the channels in the slow inactivated state, as shown below.

**Time Course of BTX Modification of the Slow Inactivated Channels in Pronase-treated Axons**

Since a long-lasting depolarization was required for BTX to modify all the channels after pronase pretreatment (Fig. 3, C and D), some BTX modification was likely to occur after the channels had entered the slow inactivated state. To test this hypothesis, we used a different protocol to promote all channels in the slow inactivated state before BTX application.

The onset and recovery from the slow inactivation were first determined in pronase-treated axons at the potential used to induce BTX modification, using a double pulse protocol. The onset kinetics followed a single exponential time course with a time constant of 4.1 s at +53 mV. After a 100-s depolarizing pulse to +53 mV, the recovery from the slow inactivation also occurred at a very slow rate, exhibiting a monoexponential time course with a time constant of 7.6 s at -120 mV. Both processes occurred on the time scale of seconds, as reported by Rudy (1978).

To promote all the channels in the slow inactivated state before BTX application, the membrane potential was maintained at the reversal potential $E_{Na}$, about +50 mV, and BTX was introduced to the internal side of the axon while the membrane was maintained depolarized (see protocol in Fig. 6 B). After repolarizing the membrane at -150 mV for 5 min, a single test pulse to -70 mV was applied, followed by a single $I_{Na-V}$ curve established to estimate the degree of BTX modification. In these conditions, exposing the axon to 4 μM BTX for 30 min induced a large inward current at -70 mV, which could be completely blocked by 1 μM TTX (see Fig. 6 A, inset). The conductance–voltage relationship was very similar to that obtained in the
same axon after the subsequent stimulation by a train of depolarizing pulses to +80 mV shown to induce complete BTX modification (Fig. 6A).

The time course of BTX modification of the slow inactivated channels was determined by using the protocol in Fig. 6B with various durations of the sustained

![Figure 6](https://example.com/f6.png)

**Figure 6.** BTX modification of slow inactivated Na channels in pronase-treated axons. (A) Voltage dependence of the normalized steady-state conductance obtained in the presence of 4 μM BTX, after 30 min depolarization to +50 mV according to the protocol shown in the inset in B with Δt = 30 min (open squares), and after the subsequent application of a train of 3,200 CP to +80 mV (filled squares). Eq. 1 with $E_{1/2} = -73.6$ mV and $k = 13.2$ mV was used to fit the filled squares (solid line). (Inset) Inward Na current recorded during the first TP to −70 mV (trace labeled BTX) after the 30-min depolarization to +50 mV (protocol shown in B with Δt = 30 min). The current trace recorded after the subsequent external application of 1 μM TTX (trace labeled TTX) is superimposed. In B, the degree of BTX modification, induced by applying the protocol in the inset in the presence of 4 μM BTX with various durations of Δt, was estimated as the shift of the midpoint potential of the conductance–voltage relationships determined with respect to the control (after washing away pronase), normalized to its maximal value obtained for complete BTX modification and plotted as a function of the depolarization duration under BTX application (Δt). Each data point corresponds to an individual axon. The solid line represents a single exponential function of time with a time constant of 9.5 min. The delay (~3 min) corresponds to the estimated time for BTX to equilibrate in the axon. (HP = −150 mV, 300 Na+/50 Na+).

depolarization under BTX internal perfusion (Δt). With such a protocol, one axon could be used once only to obtain one data point. The degree of BTX modification was evaluated for each axon from the extent of the shift of the midpoint of the $g_{Na}V$ curves with respect to the control, normalized to the maximal shift elicited by...
repetitive stimulation (same protocol as in Fig. 2 B) which shifted the potential of half-maximal conductance from \(-11 \pm 2\) mV (\(N = 5\)) after pronase treatment to \(-74 \pm 4\) mV (\(N = 5\)) after complete BTX modification. The fraction of this maximal shift obtained for six different durations of the depolarization under BTX application is plotted as a function of time in Fig. 6 B. A 30-min depolarization caused a nearly complete modification (97.5\%) as illustrated in Fig. 6 A. The onset of BTX modification of the slow inactivated channels could be approximated by a single exponential time course, with a time constant of 9.5 min. These data demonstrate unequivocally that, after the removal of the fast inactivation, BTX could modify virtually all the channels in the slow inactivated state, but much more slowly than the open channels.

**DISCUSSION**

The main goal of this study was to determine the mechanism of BTX-induced modification in squid axons. We demonstrate here that BTX modification is state dependent and can occur through two different pathways. First, for the channels that inactivate in the control, all BTX modification occurs via the open state. Second, for the channels that lack the fast inactivation, spontaneously or pharmacologically, BTX can modify both the open state and the slow inactivated state. The relative rate constants of modification of the open state and slow inactivated state will be first estimated, before discussing the pathways of BTX modification in different experimental conditions.

**Rate of Modification of the Open State**

The following calculations are based on two main assumptions. First, the channels are modified by BTX during the depolarizing pulse. Second, the steady-state current (or late current) in control axons is due to noninactivating channels behaving as pronase-treated channels, with a mean open time limited by the time constant of the slow inactivation. This second assumption is based on the single-channel analysis of the late Na current by Patlak and Ortiz (1986, 1989), who showed that, in skeletal muscle fibers, most of the late current is due to channels that do not inactivate for extended periods during maintained depolarizations, entering in a prolonged (100–200 ms) bursting mode with a fractional open time close to 100\% at positive potentials. In the absence of equivalent analysis in squid axon, we will simply assume, by analogy, similar properties for squid Na channels.

The rate of BTX modification of the open state was first estimated in pronase-treated axons from the time course of modification induced by a train of repetitive 4-ms depolarizations to +80 mV (Fig. 4 A). With a time constant of the onset of the slow inactivation of \(\sim 4\) s after pronase treatment, nearly 100\% of the channels remain open during a 4-ms depolarizing pulse to this potential. Therefore, one can assume that the rate of BTX modification corresponds to the rate of modification of the open state. The time constant of modification of the open state, \(\tau_o\), was calculated directly from the time constant of BTX modification expressed in terms of number of pulses (\(\tau(n) = 314 \pm 29\) pulses), as \(\tau_o = \tau(n) \times 4\) ms, giving \(1.3 \pm 0.1\) s (\(N = 2\)). Increasing the duration of the conditioning pulse from 4 to 100 ms markedly accelerated the rate of BTX modification (Fig. 4 B), decreasing the averaged time constant \(\tau(n)\) to \(12 \pm 4\) pulses (\(N = 2\)). Since 98\% of the channels are still open by the end of a 100-ms pulse (same calculation as above), the onset of BTX modification still
represents the rate of modification of the open channels. A similar calculation as above gives a time constant of modification of \(1.2 \pm 0.4\) s \((N = 2)\). These calculated time constants of modification of the open state are close to the experimental ones of 1.3 and 0.90 s determined from the onset of BTX modification as a function of cumulative durations of the conditioning pulses for the 4- and 100-ms pulse protocols, respectively (Fig. 5).

In control axons, the fast inactivated channels were not modified by BTX (Fig. 3). Since a fraction of the channels becomes fast inactivated during each depolarization, the time constant of BTX modification depends on the fraction of open channels, the mean open time of the inactivating and noninactivating channels, and the rate of modification of the open state. The rate of modification of the open state was first determined from the time constant of modification during a train of 4-ms depolarizing pulses to +80 mV (Figs. 2 B and 4A), using the integral of the Na current over a 4-ms depolarization as an estimate of the total availability of open channels for BTX modification. The proportion of the inactivating channels, \(51 \pm 6\%\) \((N = 5)\), was calculated from the difference between the averaged integrals of the Na current before and after the removal of the fast inactivation by pronase, and the open time of these channels was assumed to equal the time constant of the fast inactivation, i.e., 0.83 ± 0.09 ms \((N = 5)\) at +80 mV. Therefore, during each 4-ms pulse the inactivating channels contribute for 0.42 ms to the total open time, whereas the noninactivating channels (49%) contribute for 1.96 ms. With a total open time of 2.38 ms, the averaged time constant of BTX modification, expressed as a number of pulses \((\tau(n) = 462 \pm 32\) pulses, \(N = 3)\), corresponds to 1.1 ± 0.2 s \((N = 3)\), a value comparable to that determined in pronase-treated axons. We conclude that indeed, in control axons, all the channels modified during a train of 4-ms depolarizing pulses are modified in the open state. However, the rate of modification of the open state is very slow as compared with the mean open time of the channel. During a single 4-ms depolarization, only 0.07% of the inactivating channels and 0.3% of the noninactivating ones become modified by BTX in our experimental conditions. As a consequence, a prolonged high frequency pulsing is required to modify all the channels (Fig. 2 B), as also observed by Khodorov and Revenko (1979) in the frog Ranvier node.

Increasing the duration of the conditioning pulse to 100 ms (Fig. 4 B) accelerated the rate of BTX modification in control axons, decreasing the time constant to 63 ± 10 pulses \((N = 2)\). The fraction of the noninactivating channels during a 100-ms pulse was estimated from the steady-state inactivation–voltage relationships, giving 20.3 ± 0.7% \((N = 7)\) at +50 mV. Since the time constant of the fast inactivation at +50 mV was 0.85 ± 0.05 ms \((N = 10)\), the inactivating channels \((\sim 80\%)\) contributed for 0.68 ms to the total open time, whereas the noninactivating ones \((\sim 20\%)\) contributed for 20 ms to the total open time. The time constant of the BTX modification calculated as above becomes 1.3 ± 0.2 s \((N = 2)\), a value resembling that calculated in pronase-treated axons. Therefore, in control axons the channels that are modified during depolarizations up to 100 ms are modified in the open state and belong mostly to the fraction of the noninactivating channels. Accordingly, in control axons, where there were about five times fewer noninactivating channels \((20\%)\) than in pronase-treated axons, the rate of BTX modification was about fivefold slower than in pronase-treated axons \((63\) vs. 12 pulses).
As summarized in Table I, the removal of the fast inactivation did not alter the rate of modification of the open state. In both control and pronase-treated axons, the averaged time constant of BTX modification of the open state was $1.22 \pm 0.08$ s ($N = 4$), giving a rate constant of $0.82$ s$^{-1}$. Since the BTX modification is essentially irreversible, the onset rate of BTX modification mainly reflects the forward rate constant. Taking into account the BTX concentration (4 μM), the forward rate constant for BTX binding to the open channels is thus $2 \times 10^5$ M$^{-1}$ s$^{-1}$.

**Rate of Modification of the Slow Inactivated State**

In pronase-treated axons, all the channels could also be modified in the slow inactivated state with a time constant of $\sim 9.5$ min (Fig. 6 B), giving a rate constant of $1.7 \times 10^{-5}$ s$^{-1}$. The same calculation as above gives a bimolecular rate constant for BTX to modify the slow inactivated channels of $4.4 \times 10^2$ M$^{-1}$ s$^{-1}$, a value $\sim 500$-fold smaller than that obtained for the open channel modification. Since the rate of modification of the open state is much faster than that of the slow inactivated state, the contribution of the open channel modification is expected to accelerate the BTX modification onset during a sustained depolarization, as experimentally observed when BTX was applied before the depolarization (cf. Fig. 3, C and D, and Fig. 6).

| Time Constants of BTX Modification of the Open State |
|------------------------------------------------------|
| 4-ms pulse protocol | 100-ms pulse protocol |
| $\tau$ (s) | $\tau$ (s) |
| Control axons | 1.1 ± 0.2 (3) | 1.3 ± 0.2 (2) |
| Pronase-treated axons | 1.3 ± 0.1 (2) | 1.2 ± 0.4 (2) |

The calculation of the rate constants is discussed in the text.

In control axons, the fast inactivation seemed to protect the slow inactivated state from BTX modification, since only slight modification ($\sim 20\%$) was observed during a 10-min depolarization to +45 mV (Fig. 3 A). With such a protocol, all the noninactivating channels ($\sim 20\%$) become modified, either the open or the slow inactivated state, as observed after pronase treatment (see above and Fig. 3 D). If all the inactivating channels ($\sim 80\%$) were also modified in the slow inactivated state at the same rate as in pronase-treated axons, another 50% of the channels would have been modified. Since the number of inactivating channels modified in the open state at the beginning of a single depolarization does not exceed 0.06% of the channels, the small degree of BTX modification observed (20%) is thus definitely due to the modification of the noninactivating channels. These results suggest that the fast inactivation still protected the channels from BTX modification after the slow inactivation had developed. Therefore, for the channels that inactivate in control axons, both the fast and the slow inactivated states are insensitive to BTX action.

**Are the Slow Inactivated Pronase-treated Channels Also Modified in the Open State?**

The estimated rate constant of BTX modification in pronase-treated axons remains unchanged for depolarizations from 4 to 100 ms, suggesting that the gating behavior
of the pronase-treated channels did not change during such depolarizations. This result is consistent with a marked prolongation of the lifetime of the channels, as reported in other preparations after the removal of the fast inactivation (Patlak and Horn, 1982; Horn et al., 1984; Quandt, 1987).

However, a marked decrease of the rate of modification from 0.8 to 0.04 s⁻¹ was observed when lengthening the duration of the depolarization from 100 ms to the order of seconds. A 1-s depolarization to +50 mV induced the modification of <10% of the channels (Fig. 5), instead of ~50% as expected from time constants of ~1 s for the open channel modification and 4 s for the onset of the slow inactivation. These results suggest a change in the gating behavior of the channels associated with a marked decrease in the total open time of the channel.

Several single-channel studies have shown that, after the pharmacological removal of the fast inactivation, the gating behavior of the treated channels indeed changes with the depolarization time, shifting from a marked prolonged open time usually observed during the first hundreds of milliseconds (Patlak and Horn, 1982; Horn et al., 1984), to a clustering of openings separated by long silent periods (hibernation) occurring on the time scale of seconds (Horn et al., 1984), and to a bursting activity separated by very long silent periods (tens of seconds) observed during chronic depolarization (Quandt, 1987).

Our results would be consistent with such an alteration of the single-channel gating behavior of the squid pronase-treated axons. If indeed the macroscopic "slow inactivation" corresponds, at the microscopic level, to a very infrequent bursting activity of the channels between the open and the closed states (Quandt, 1987), the macroscopic time constant of the slow inactivation during sustained depolarization would be determined by the burst duration rather than by the mean open time of the channels, as shown by Quandt (1987) in neuroblastoma cells. In this case the channels could be modified during the interburst interval and/or in the open state within the burst. If the channels are modified only in the open state during the burst at the same rate as calculated above, the total open time should not exceed 0.2% of the total depolarization time, since, at the macroscopic level, the rate of modification of the slow inactivated state is 500-fold slower than that of the open state. In such a hypothesis, all BTX modification would take place via open channels, as proposed in the open channel hypothesis, but in different gating modes.

The final interpretation of these data would require a characterization of the single-channel properties of the slow inactivated Na channels in squid pronase-treated axons. Since the macroscopic measurements cannot discriminate between a modification within the burst and during the interburst interval, we will still define the modification occurring during maintained depolarization as the slow inactivated state modification. In conclusion, the channels that inactivate in the control can be modified only in the open state, whereas the channels that lack the fast inactivation can be modified in both the open state and the slow inactivated state.

Comparison with Previous Work

The present results first substantiate the hypothesis previously proposed for BTX action in the frog Ranvier node by Khodorov and Revenko (1979), who concluded that the opening of the Na channels seems to be a prerequisite for the BTX
modification, and that the receptor for BTX seems to be inaccessible or absent in the resting or inactivated channels. Second, our results are consistent with the open channel modification hypothesis for the alkaloid neurotoxins proposed by Hille (Hille, 1984; Hille et al., 1987), but unveil a second pathway that can account for the modification observed with other alkaloid neurotoxins under maintained depolarizations. The relative contribution of these two pathways to BTX modification, which depends on the membrane potential and on the relative fraction of the inactivating and noninactivating channels, is expected to vary from one preparation to another.

Such a state dependence of BTX action can account for several findings in previous studies on BTX action. First, in squid axons the application of BTX at the resting potential in the absence of repetitive stimulation fails to induce a significant modification of the Na current, while it causes a large membrane depolarization (Narahashi et al., 1971; Albuquerque et al., 1973). At the resting potential, the low probability of the opening of the channels allows only a small fraction of the channels to be modified. Since the BTX-modified channels do not undergo the slow inactivation in squid axons (unpublished personal data), this small fraction of modified channels produces a maintained membrane depolarization, which induces the fast inactivation of the unmodified inactivating Na channels, rendering them insensitive to BTX action and thus preventing further modification of a larger number of Na channels even during a prolonged application of BTX. Simultaneously, the depolarization promotes the modification of the small fraction of the noninactivating channels, which produces a further membrane depolarization without inducing a significant shift of the activation voltage dependence (cf. Fig. 3 B).

Second, the insensitivity of the fast inactivated state to BTX modification could explain the antagonizing effect on BTX action of agents that stabilize the fast inactivated state, such as local anesthetics or antiarrhythmic drugs, as observed in electrophysiological and binding studies (Albuquerque et al., 1973; Huang et al., 1978; Khodorov, 1978; Catterall, 1981, 1987; Willow and Catterall, 1982; Creveling et al., 1983; Soldatov et al., 1983).

Third, under chronic depolarization the pathway of modification via the slow inactivated state of the channels that lack the fast inactivation is likely to play an important role in the potentiation of BTX action by agents that remove the fast inactivation without removing the slow one. For example, the *Leiurus* α-scorpion toxin (α-ScTx) could potentiate BTX action in depolarized synaptosomes (Tamkun and Catterall, 1981) by increasing the number of channels modifiable by BTX in the slow inactivated state. The long incubation time (30–60 min) required for BTX binding in such conditions supports this interpretation. According to the general allosteric model of Catterall (1977), α-ScTx favors the binding of the agonist BTX (or other alkaloid toxins) to an “active,” conducting form of the channel for which BTX has a higher affinity than for an “inactive,” nonconducting form. Our results indicate first, that the closed states and the fast and slow inactivated states cannot be lumped together into the inactive form, and second, that the active form can represent either the open state or the slow inactivated state of the channels lacking the fast inactivation, depending on the membrane potential.

Among the other alkaloid neurotoxins, the modification pathway has been characterized only for veratridine. Hille and his collaborators have clearly established that
the veratridine-induced modification occurs from the open state in skeletal muscle fibers and in neuroblastoma cells (Leibowitz et al., 1986, 1987; Sutro, 1986; Barnes and Hille, 1988). The modification is use dependent, requiring repetitive stimulation, and the rate constant of modification of the open state is not altered by agents that remove the fast inactivation, such as NBA, chloramine-T, and Leuurus scorpion toxin (Sutro, 1986). Veratridine modifies the channels from the open state with a bimolecular rate constant of $2 \times 10^6 \text{M}^{-1} \text{s}^{-1}$ (Leibowitz et al., 1986; Barnes and Hille, 1988), i.e., at a rate $\sim 10$-fold faster than BTX. All these features of the open channel modification are similar to those reported here for BTX. Although the relative roles of the fast and slow inactivation were not specifically addressed in these studies, Sutro (1986) concluded that the rate of modification of the inactivated channels by veratridine was $<1.5\%$ of the rate of modification of the open channels, suggesting that the binding of veratridine to the inactivated channels, if any, might be very slow. These results are not fundamentally different from our finding that in control axons BTX does not modify the fast inactivated channels. In contrast, reinvestigating the action of veratridine in the frog Ranvier node, Rando (1989) proposed that veratridine also modifies the channels in the fast inactivated state in order to explain the kinetics and voltage dependence of the slowly activating current that develops over several seconds during maintained depolarizations (Ulbricht, 1969). However, Hille and collaborators (Hille et al., 1987) have shown that the binding of veratridine to the open channel could also account for the slow development of the modified current during prolonged depolarization, reflecting the binding of veratridine to the channels that spontaneously, but infrequently, reopen from the inactivated state.

In conclusion, the main pathway of BTX modification via the open channels seems to be common for the alkaloid neurotoxins. The second pathway of BTX modification via the slow inactivated state of channels lacking the fast inactivation, which has not yet been investigated for the other alkaloid toxins, is probably negligible in physiological conditions, but might become predominant under chronic depolarizations in membranes with a significant fraction of Na channels lacking the fast inactivation process, occasionally, pharmacologically, or genetically.

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