Inactivation of Batrachotoxin-modified Na^+ Channels in GH_3 Cells

Characterization and Pharmacological Modification

GING KUO WANG and SHO-YA WANG

From the Department of Anesthesia Research Laboratories, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115; and Department of Biology, State University of New York at Albany, Albany, New York 12222

ABSTRACT Batrachotoxin (BTX)-modified Na^+ currents were characterized in GH_3 cells with a reversed Na^+ gradient under whole-cell voltage clamp conditions. BTX shifts the threshold of Na^+ channel activation by ~40 mV in the hyperpolarizing direction and nearly eliminates the declining phase of Na^+ currents at all voltages, suggesting that Na^+ channel inactivation is removed. Paradoxically, the steady-state inactivation (h_∞) of BTX-modified Na^+ channels as determined by a two-pulse protocol shows that inactivation is still present and occurs maximally near −70 mV. About 45% of BTX-modified Na^+ channels are inactivated at this voltage. The development of inactivation follows a sum of two exponential functions with $\tau_{d(fast)}$ = 10 ms and $\tau_{d(slow)}$ = 125 ms at −70 mV. Recovery from inactivation can be achieved after hyperpolarizing the membrane to voltages more negative than −120 mV. The time course of recovery is best described by a sum of two exponentials with $\tau_{r(fast)}$ = 6.0 ms and $\tau_{r(slow)}$ = 240 ms at −170 mV. After reaching a minimum at −70 mV, the h_∞ curve of BTX-modified Na^+ channels turns upward to reach a constant plateau value of ~0.9 at voltages above 0 mV. Evidently, the inactivated, BTX-modified Na^+ channels can be forced open at more positive potentials. The reopening kinetics of the inactivated channels follows a single exponential with a time constant of 160 ms at +50 mV. Both chloramine-T (at 0.5 mM) and α-scorpion toxin (at 200 nM) diminish the inactivation of BTX-modified Na^+ channels. In contrast, benzocaine at 1 mM drastically enhances the inactivation of BTX-modified Na^+ channels. The h_∞ curve reaches a minimum of <0.1 at −70 mV, indicating that benzocaine binds preferentially with inactivated, BTX-modified Na^+ channels. Together, these results imply that BTX-modified Na^+ channels are governed by an inactivation process.

INTRODUCTION

Batrachotoxin (BTX) is a steroidal alkaloid known to modify both the Na^+ channel activation and inactivation gating processes (for review, see Khodorov, 1978; Hille,

Address reprint requests to Dr. Sho-Ya Wang, Department of Biology, SUNY at Albany, Albany, NY 12222.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/92/01/0001/20 $2.00 1
Volume 99 January 1992 1–20

1984). In general, BTX drastically shifts the threshold of Na^+ channel activation. Normally, Na^+ channels are activated at about −40 mV, whereas BTX-modified channels are activated at about −80 mV. In addition, during a prolonged depolarization BTX-modified Na^+ channels give rise to a maintained Na^+ current that does not inactivate. Other characteristics of Na^+ channels are also altered by BTX: (*a*) the ion selectivity of Na^+ channels is decreased (Khodorov, 1978); (*b*) the voltage-dependent binding of tetrodotoxin (TTX) with the Na^+ channel becomes evident (Krueger et al., 1983); (*c*) the stereoselectivity of local anesthetics (LAs) with the Na^+ channel is changed (Wang, 1990); and (*d*) the binding affinity of α-scorpion toxin is enhanced (Catterall, 1980). Clearly, BTX upon binding can alter the Na^+ channel gating properties as well as the dimension of the ion pathway and the conformation of the channel external surface (for review, see Strichartz et al., 1987).

We have become interested in the interactions of BTX-modified Na^+ channels and LAs because it has been shown that BTX reduces the binding affinity of many LAs to the Na^+ channel receptor (Khodorov, 1978; Rando et al., 1986). However, some LAs remain relatively effective in blocking BTX-modified Na^+ channels in bilayers (Wang, 1988) as well as in nerve fibers (Khodorov, 1978). The reason for this differential reduction of LA binding by BTX is unknown. One possibility is that some LAs preferentially bind with open Na^+ channels, whereas others bind primarily with the inactivated channels.

Hille (1977) found that benzocaine, a neutral LA, is able to promote Na^+ channel inactivation upon binding. He reported that benzocaine shifts the steady-state inactivation (h_∞) curve by −25 mV (in the hyperpolarizing direction). Hille interpreted that more Na^+ channels are inactivated because benzocaine preferentially binds and subsequently stabilizes the inactivated state of Na^+ channels. The state-dependent binding of LAs with Na^+ channels is now known as the "modulated receptor hypothesis" (Hille, 1977).

Assuming that BTX indeed removes Na^+ channel inactivation, we predicted that BTX would also significantly reduce the binding affinity of benzocaine. To our surprise, we found that this is not the case in GH_3 cells. At the "resting" potential (i.e., −70 mV) benzocaine is more potent in BTX-modified Na^+ channels ($K_d \approx 0.13$ mM) than in normal Na^+ channels ($K_d \approx 1.3$ mM) by a factor of 10. This result prompted us to reexamine the assumption that Na^+ channel inactivation is eliminated by BTX. Contrary to the general belief, our study shows that Na^+ channel inactivation can occur in BTX-modified Na^+ channels. Although a similar inactivation phenomenon of BTX-modified Na^+ channels was briefly stated before (Zubov et al., 1984; Mozhayeva et al., 1986), Dubois and Coulombe (1984) concluded that sodium accumulation and depletion in the perinodal space of myelinated fibers is the reason for the apparent inactivation in BTX-treated Na^+ channels. In this report we have used a pharmacological approach to detail this inactivation phenomenon in GH_3 cells. Since GH_3 cells do not have a perinodal barrier, sodium ion accumulation and depletion in this preparation are limited. Continuous superfusion of GH_3 cells with Na^+-free solution also minimizes changes of Na^+ reversal potential during experiments. Finally, under reversed Na^+ gradient conditions there is no inward Na^+ current and hence no internal Na^+ ion accumulation. The ramifications of the

presence of inactivation on the kinetic modeling and on the action of LAs in BTX-modified Na^+ channels will be discussed.

MATERIALS AND METHODS

Chemicals

Benzocaine was purchased from Sigma Chemical Co. (St. Louis, MO). Chloramine-T (CT) was obtained from Aldrich Chemical Co. (Milwaukee, WI). α-Leiurus toxin (L.Q. IIα) was purified by cation column chromatography as described (Wang and Strichartz, 1983). BTX was a generous gift from Dr. John Daly, NIH (Bethesda, MD). TTX was obtained from Calbiochem Corp. (La Jolla, CA). All other chemicals were reagent grade from commercial sources without further purification.

Cell Culture

GH_3 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in DMEM supplemented with 10% fetal bovine serum and 1% glutamine (Gibco Laboratories, Grand Island, NY) essentially as described by Cota and Armstrong (1989). Cells were plated on 35-mm plastic petri dishes (Falcon Plastics, Cockeysville, MD). Experiments were usually carried out 3 d after plating, with cell density ~30–60% confluent.

Whole-Cell Voltage Clamp

The whole-cell variant of the patch clamp method (Hamill et al., 1981) was used to measure Na^+ currents in GH_3 cells. The petri dish that contained GH_3 cells was first rinsed with an external solution containing (mM): 150 choline·Cl, 0.2 $CdCl_2$, 2 $CaCl_2$, and 10 HEPES adjusted to pH 7.4 with TMA-OH. The dish with GH_3 cells was then used as a recording chamber (containing ~0.5 ml external solution) which was continuously perfused with the external solution at a flow rate of 1 ml/min.

Micropipettes were fabricated from borosilicate capillary tubing and had tip resistances of ~1 MΩ when filled with an internal high Na^+ solution containing (mM): 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES adjusted to pH 7.2 with CsOH. The advantages of using high internal Na^+ ions have been discussed before (Cota and Armstrong, 1989). Under this reversed Na^+ gradient condition, outward I_{Na} is easily visible at all voltages and the voltage error caused by access resistance is less serious with outward than with inward I_{Na}. There is no evidence that external choline inhibits Na^+ currents in control or internally papain-treated GH_3 cells (Cota and Armstrong, 1989). Except for BTX, drugs were applied to cells via a series of narrow bored capillary tubes positioned to within 200 μm of the cell. To minimize the consumption of BTX this drug was applied intracellularly within the micropipette at a final concentration of 5 μM. BTX in the micropipette did not affect the formation of a GΩ seal with GH_3 cells. The holding potential was set at −100 mV in all experiments.

Data Acquisition and Analysis

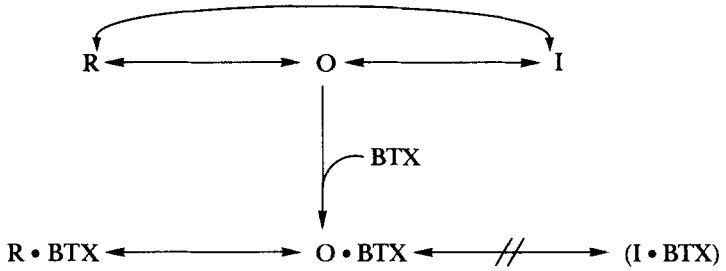
The recording system consisted of a List EPC-7 patch clamp amplifier (Medical Systems, Corp., Greenvale, NY), a home-made leak and capacitance subtractor, and an IBM-AT computer interfaced to the amplifier by a 100-kHz Labmaster board (Scientific Solutions Inc., Solon, OH). Creation of voltage clamp pulses, data acquisition, and data analysis were performed using pClamp software (Axon Instruments, Inc., Foster City, CA). On some occasions, leak and capacitance were further subtracted by P/-4 protocol, but this procedure was omitted whenever possible because of its possible effect on benzocaine binding. Series resistance was compensated

by using a List EPC-7 device; ~40–80% of series resistance was compensated. The estimated maximal voltage error created by series resistance at +50 mV is about ≤5 mV. Curve fitting was performed using the Levenberg-Marquardt algorithm (Marquardt, 1963). Results are expressed as mean ± SE.

RESULTS

General Description of BTX Effects on Na^+ Currents in GH_3 Cells

The action of BTX on the kinetics of Na^+ currents has been well studied in various preparations, although not in GH_3 cells. Briefly, Na^+ currents in GH_3 cells normally appear when the membrane is depolarized to more positive than −40 mV. Under reversed Na^+ gradient conditions, the outward Na^+ current rises to reach its peak, and then declines to near zero current level (Fig. 1 *A*; also see Cota and Armstrong, 1989). Without external Na^+ ions present the peak current–voltage relationship is almost linear (Fig. 1 *C*, open circles). When 5 μM BTX was included in the patch pipette, a small, sustained Na^+ current could be detected during a prolonged depolarization after the break-in of the pipette. This sustained current grew to more than half of the peak Na^+ current amplitude after a 10-min continuous stimulation of cells with repetitive pulses (50 mV/50 ms at a frequency of 2 Hz; Khodorov, 1978). Nearly all the normal and BTX-modified Na^+ currents could be blocked by 100 nM TTX (data not shown). Fig. 1 *B* shows that the BTX-modified Na^+ currents are maintained during prolonged depolarizations and the peak current–voltage relationship reveals that BTX-modified channels exhibit an activation threshold at about −90 mV (Fig. 1 *C*; closed circles) or ~40 mV more negative than the unmodified counterparts (Fig. 1 *C*, open circles). At the time scale used in Fig. 1 *B*, the unmodified Na^+ currents were not recorded due to their fast inactivating kinetics. Our results on BTX-modified Na^+ channels in GH_3 cells thus agree well with previous reports using various cell preparations (e.g., Huang et al., 1987; Tanguy and Yeh, 1988) and suggest a binding of BTX to open Na^+ channels as follows:



Scheme 1

where R, O, and I represent resting, open, and inactivated normal Na^+ channels, respectively; R·BTX, O·BTX, and I·BTX represent BTX-bound resting, open, and inactivated Na^+ channels, respectively. BTX appears to bind preferentially to the open channel and upon binding it shifts the R·BTX → O·BTX transition by about −40 mV. The fast inactivation O·BTX → I·BTX transition is thought to be abolished

since the BTX-modified Na^+ currents are maintained during a prolonged depolarization.

Steady-State Inactivation of BTX-modified Na^+ Channels

Steady-state inactivation (h_∞) was measured by a two-pulse protocol. As in nerve fibers (Hodgkin and Huxley, 1952), the steady-state level of Na^+ channel inactivation in

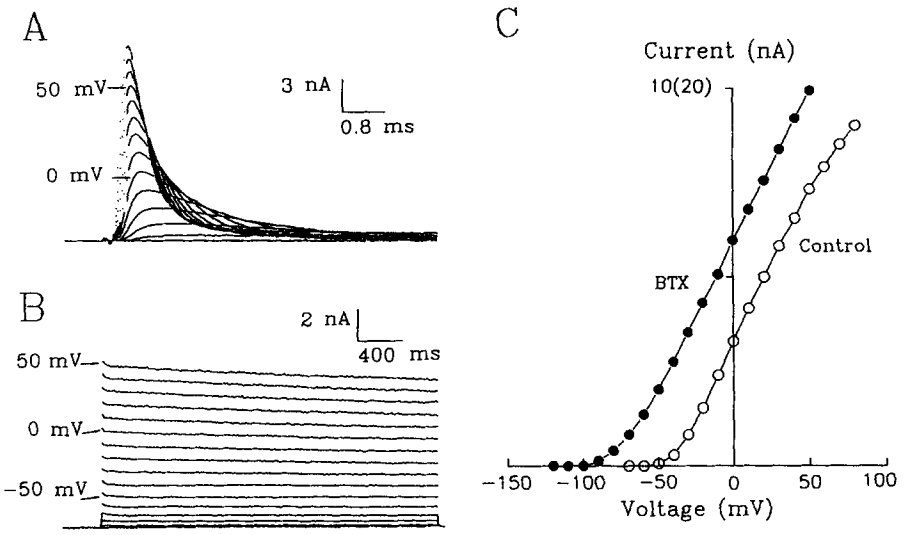


FIGURE 1. Na^+ currents in GH_3 cells under a reversed Na^+ gradient. (*A*) Na^+ currents without BTX at various test voltages were superimposed in the figure. Leak and capacitative currents were subtracted. Step depolarizations from −70 to +80 mV with a 10-mV increment were used to elicit Na^+ currents. The current traces of 0 and 50 mV are labeled. The holding potential was set at −100 mV and a hyperpolarizing prepulse of −130 mV for 50 ms was applied before each test pulse. (*B*) BTX-modified Na^+ currents at various test voltages were superimposed in the figure. The record was taken after repetitive stimulations of the cell for ~10 min. Notice the slow time scale used in the recording (10 ms/point); unmodified Na^+ currents were too fast to be recorded at this time scale. BTX-modified Na^+ currents were elicited by 10-mV step depolarizations from −120 to +50 mV. A hyperpolarizing prepulse of −130 mV for 3 s was applied before the test pulse. The pipette solution contained 5 μM BTX. (*C*) The peak BTX-unmodified and BTX-modified Na^+ currents were measured from *A* (*open circles*) and *B* (*closed circles*), respectively, and plotted against voltages. Notice that the current scale of BTX is half of the control in the *y* axis. Data for control and BTX-modified currents were from two separate GH_3 cells.

GH_3 is voltage dependent. This is shown in Fig. 2 *A*, where the peak Na^+ current at the test potential (+30 mV) is highly sensitive to the prepulse membrane potential. The normalized peak Na^+ current amplitudes were plotted against the prepulse potential. The midpoint of the curve is measured at −70 ± 3 mV (n = 3), where 50% of peak Na^+ current is inactivated (Fig. 2 *C*, open circles).

In contrast, the effect of the prepulse on the BTX-modified Na^+ channels is complicated. Fig. 2 *B* shows the superimposed Na^+ currents generated by a +50-mV test pulse (E_t) with two different prepulse potentials. With a −170-mV prepulse of 3-s duration, the BTX-modified Na^+ current rises rapidly to reach its peak and declines slightly thereafter (trace *1*). With a −70-mV prepulse, the rising phase of Na^+ current

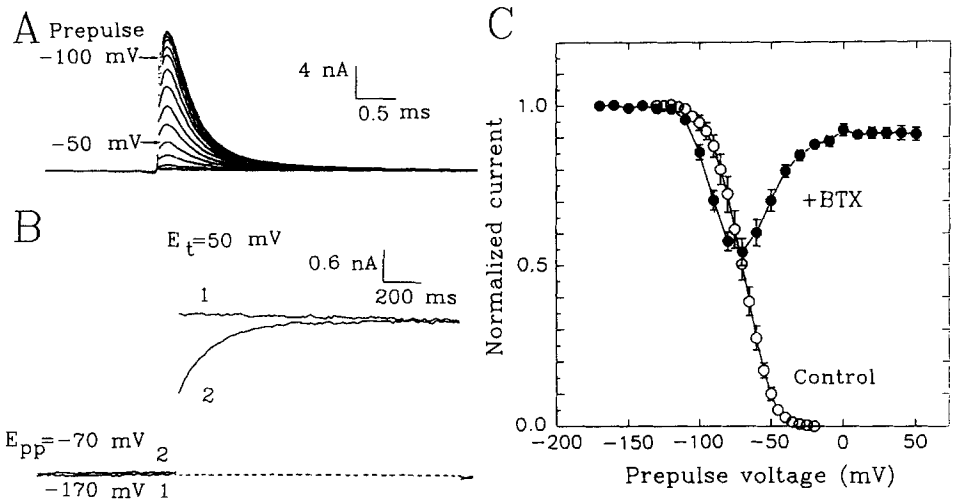


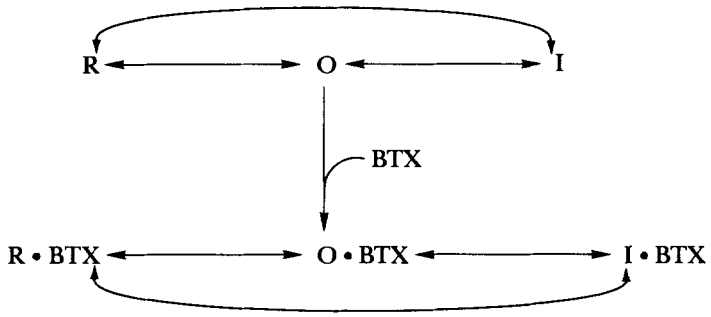
FIGURE 2. Steady-state Na^+ channel inactivation in control and BTX-treated GH_3 cells. (*A*) Steady-state Na^+ channel inactivation in the absence of BTX was measured by a two-pulse protocol. Current traces at the test voltage of +30 mV were superimposed in the figure. Prepulses from −130 to −20 mV for 50 ms with a 10-mV increment were applied before the test pulse. The holding potential was set at −100 mV. Current traces with prepulses of −100 and −50 mV are labeled. (*B*) Steady-state inactivation in the presence of 5 μM internal BTX was also measured by a two-pulse protocol. Superimposed were current traces at the test voltage of +50 mV with prepulse voltages of −170 mV (trace *1*) and −70 mV (trace *2*) for 5 s. The holding potential was set at −100 mV. Notice the different time scales for *A* and *B*. (*C*) Peak Na^+ currents at the test pulse with various prepulse voltages as in *A* (*open circles*) were normalized and plotted (h_∞ curve). The data of open circles were least-squares fitted with an empirical Boltzmann equation, $y = 1/\{1 + \exp[(E_{pp}-E_{0.5})/k]\}$, where $k = 9.88$ and $E_{0.5} = -70$ mV (*solid line*). Data of open circles were pooled from three separate cells and all control peak currents were normalized with respect to the peak current amplitude with the −130-mV prepulse voltage. In contrast, the amplitude of BTX-modified Na^+ currents at each test pulse in *B* was measured at the first 15–30 ms (*closed circles*) where all unmodified Na^+ channels were inactivated. The prepulse duration was 5 s. Data of closed circles (mean ± SE, $n = 7$) were pooled from seven separate GH_3 cells and currents were normalized by the largest current amplitude, usually with a −170-mV prepulse.

is biphasic: it first rises as rapidly as that with a −170-mV prepulse (the rapid time course cannot be recorded with this recording time scale), but is then followed by a second slowly rising phase. The two current traces eventually overlap at the end of the prolonged test pulse. It should be noted here that a −70-mV prepulse does not promote binding of BTX with unmodified Na^+ channels since this prepulse does not

reach the activation threshold of unmodified Na^+ channels. Even at a +50-mV test pulse the additional BTX binding is $\ll 1\%$ since >600 pulses (50 mV/50 ms) were needed before the first recording. Thus, the difference in current kinetics (Fig. 2 *B*) cannot be due to the binding interaction of BTX with unmodified Na^+ channels.

One simple interpretation of the biphasic current records is that BTX-modified Na^+ channels are inactivated by $\sim 45\%$ during the −70-mV prepulse. The inactivated Na^+ channels can be slowly reactivated at larger potentials, provided that the inactivated state of BTX-modified Na^+ channels is no longer an absorbing state. The steady-state inactivation can then be measured with various prepulse potentials by assuming that the second rising phase of the current represents the reopening of inactivated channels. Fig. 2 *C* (closed circles) shows the steady-state Na^+ channel inactivation in the voltage range from −170 to +50 mV. The h_∞ curve starts to decrease at around −120 mV, reaches its minimum around −70 mV, and then turns upward to ~ 0.9 at voltages ≥ 0 mV.

The results of Fig. 2 suggest that Scheme 1 now requires an inactivated state of BTX-modified Na^+ channels as follows:



Scheme 2

where the O·BTX ↔ I·BTX transition can proceed but differs from normal O ↔ I transition. This state diagram of Scheme 2 will be substantiated by the experiments described below.

Reopening Kinetics from the Inactivated State

Assuming that the second rising phase of the BTX-modified Na^+ current is due to the reopening of inactivated channels populated during a −70-mV conditioning prepulse, we measured the kinetics of this reopening process. Fig. 3 *A* shows the current traces of the slowly rising current component at three different voltages. The slowly rising phase of Na^+ currents can be well described by a single exponential function. The kinetics of the reopening process is voltage dependent; the time constant decreases as the test voltage is decreased (Fig. 3 *B*). If this trend continues, it predicts that the reopening time constant at −70 mV is on the order of 20 ms. These data thus demonstrate that the I·BTX ↔ O·BTX transition in Scheme 2 can occur. The reopening of the inactivated, BTX-modified Na^+ channels could explain the fact that Na^+ currents are still maintained during a prolonged depolarization.

Recovery from Inactivation

Based on the h_∞ curve in Fig. 2 *C*, it is evident that little inactivation occurs at voltages less than −130 mV. Fig. 4 *A* shows a pulse protocol designed to measure the time course of recovery when a fraction of the BTX-modified Na^+ channels was first inactivated at −70 mV followed by a −170-mV interpulse with various durations. The normalized current amplitude at the +50-mV test pulse shows that BTX-modified Na^+ channels recover from their inactivation with a time course that is best described

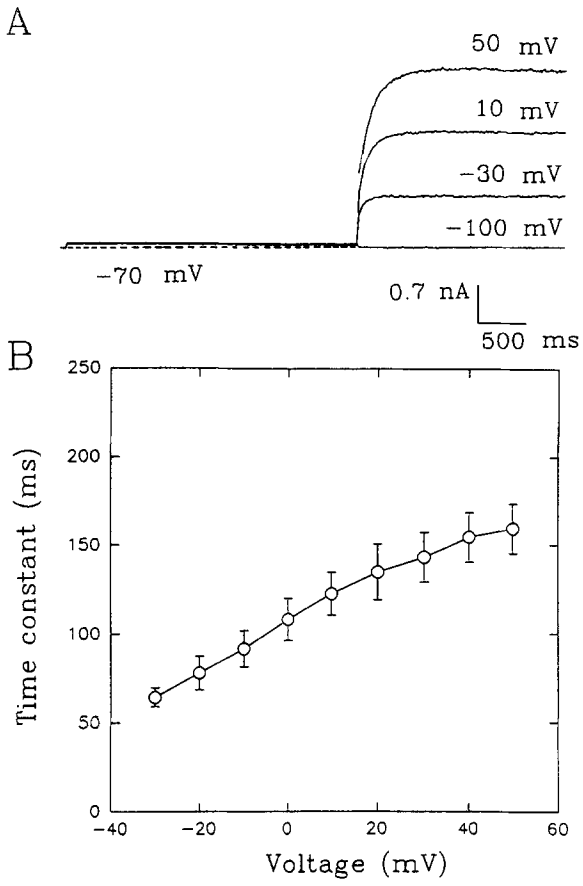


FIGURE 3. Reopening kinetics of the inactivated BTX-modified Na^+ channels. (*A*) A fraction of resting BTX-modified Na^+ channels were first inactivated at −70 mV for 3.5 s and then reactivated at various test pulses as indicated. All current traces were superimposed. The dashed line represents the zero current baseline. (*B*) Voltage dependence of the reopening time constant. The reopening current trace at each test pulse as in *A* can be well fitted with a single exponential function: $I = a[1 - \exp(-t/\tau_1)] + C$ (least-square residual, $R \geq 0.98$). The fitted time constant was plotted against the test pulse voltage. Each data point was an average value (mean ± SE) from five separate experiments.

by two exponentials (Fig. 4 *A* and inset, solid fitted line). This result demonstrates that the I·BTX ↔ R·BTX transition in Scheme 2 is present. The voltage dependence of the fast and slow time constants is shown as open circles in Fig. 5, *A* and *B*, respectively.

Development of Inactivation

The time course of the development of inactivation at −70 mV was measured by a pulse protocol as shown in Fig. 4 *B*. All BTX-modified Na^+ channels were first

permitted to enter their resting state after the membrane potential was hyperpolarized to −170 mV for 5 s, and a fraction of channels was then inactivated with various durations of a conditioning interpulse (i.e., −70 mV). The degree of inactivation was then measured at a test pulse of +50 mV. The time course of the development of inactivation can be best described by two exponentials (Fig. 4 *B* and inset, solid line).

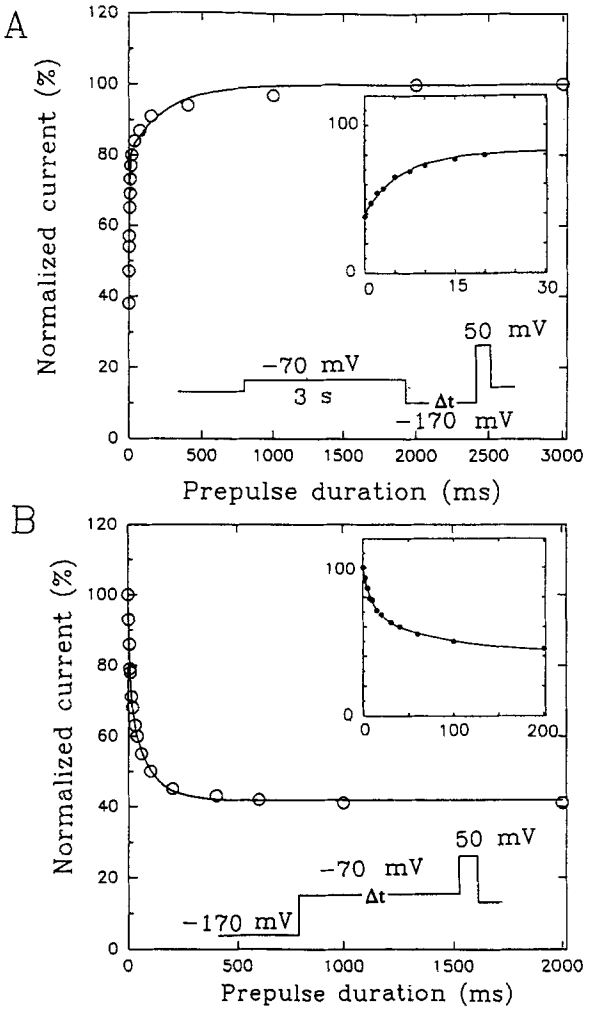


FIGURE 4. Recovery from and development of inactivation in BTX-modified Na^+ channels. (*A*) The time course of the recovery from inactivated BTX-modified Na^+ channels at −170 mV was determined by a pulse protocol shown in the figure. Currents were measured at 15–30 ms of the test pulse. The data were poorly fitted by a single exponential function. Two exponential functions of $y = a[1 - \exp(-t/\tau_1)] + b[1 - \exp(-t/\tau_2)] + c$, on the other hand, can fit the data quite well with $a = 40$, $b = 20$, $c = 40$, $\tau_1 = 5.8$ ms, and $\tau_2 = 253$ ms (*solid line*). The data with a fast time course are shown in inset (*closed circles*). (*B*) The time course of the development of inactivation was determined by a pulse protocol shown in the figure. The data were best fitted with two exponential functions: $y = a\ [\exp(-t/\tau_1)] + b\ [\exp(-t/\tau_2)] + c$, where $a = 30$, $b = 28$, $c = 42$, $\tau_1 = 9.1$ ms, and $\tau_2 = 83.3$ ms. The data with a fast time course are shown in inset (*closed circles*).

The voltage dependence of these two time constants is shown in Fig. 5, *A* and *B*, respectively (solid circles).

Unfortunately, the kinetic information regarding the recovery from and development of inactivation cannot be assigned to a specific state transition in Scheme 2 because each state represents a lumped kinetic model which may contain more than one state transition. Other reasons mentioned in the Discussion also prevent us from deriving a specific kinetic scheme at this time.

Effect of CT on Steady-State Inactivation of BTX-modified Na^+ Channels

The above descriptions of the inactivation of BTX-modified Na^+ channels raise an interesting question concerning the nature of this phenomenon. Is it a novel gating induced by BTX or is it an intrinsic inactivation process now modified by BTX? To address this question we chose a pharmacological approach.

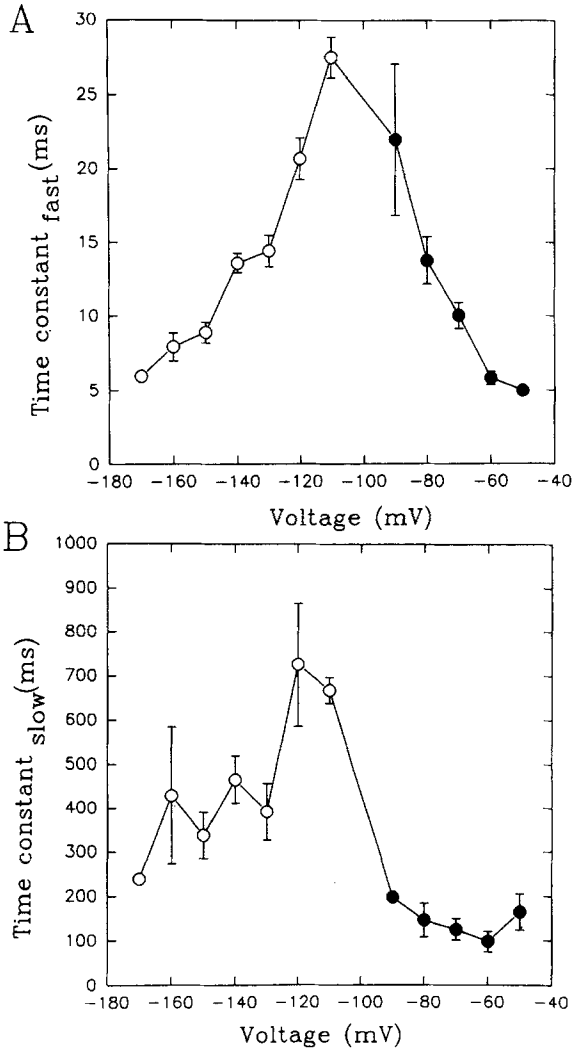


FIGURE 5. Voltage dependence of the time constants of the recovery and development of inactivation in BTX-modified Na^+ channels. The time courses of the recovery and development of inactivation as shown in Fig. 4 were determined at various interpulse voltages and fitted with two exponential functions. The fast (*A*) and slow (*B*) time constants of recovery (*open circles*) and development (*closed circles*) of inactivation are plotted against voltages ranging from −170 to −50 mV. These time constants follow a bell-shaped curve and have a peak around −120 to −110 mV.

CT, a mild oxidant, has been shown to inhibit the normal Na^+ channel inactivation in nerve fibers (Wang, 1984). In GH_3 cells, the steady-state inactivation of BTX-modified Na^+ channels was markedly altered by the external CT treatment (Fig. 6 *A*, upper trace). The degree of inactivation is slowly removed by CT over a time course

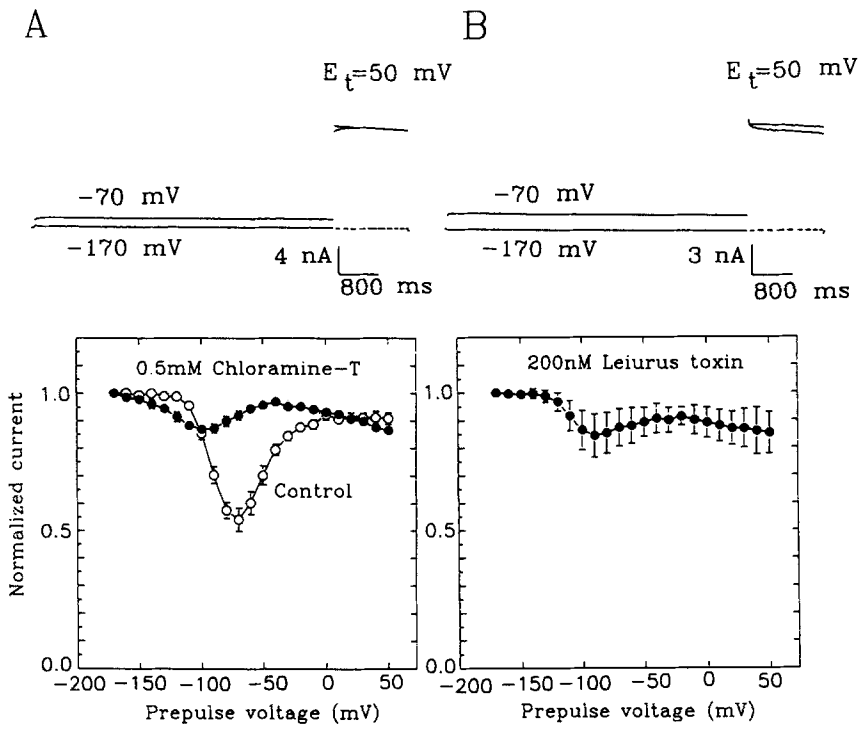


FIGURE 6. Effects of CT and α-scorpion toxin on the steady-state inactivation of BTX-modified Na^+ channels. (*A, upper trace*) Removal of inactivation of BTX-modified Na^+ channels is evident after external 0.5 mM CT treatment for 25 min. Current traces were recorded as shown in Fig. 2 *B*. Very little Na^+ current is contributed by the reopening of the inactivated, BTX-modified Na^+ channels at −70 mV during the test pulse. (*A, lower panel*) Steady-state inactivation of BTX-modified Na^+ channels is diminished by 0.5 mM external CT. Normalized BTX-modified Na^+ currents with various prepulse voltages were plotted (*solid circles*). Data were obtained by the procedure described in Fig. 2 *B* and pooled from four separate GH_3 cells. The control values in the absence of CT (*open circles*) were replotted from Fig. 2 *C* for comparison. (*B, upper trace*) BTX-modified Na^+ currents at +50 mV with prepulses of −170 and −70 mV were recorded after external 200 nM α-scorpion toxin (L.Q.IIα) treatment for 10 min. Little inactivation occurred during a −70-mV prepulse. (*B, lower panel*) Steady-state inactivation of BTX-modified Na^+ channels is reduced by 200 nM α-scorpion toxin. Normalized BTX-modified Na^+ currents were measured as in *A* and plotted against prepulse voltages. Data were pooled from three separate GH_3 cells.

of 30 min. After 30 min, only ~10% of inactivation occurs at −70 mV (Fig. 6 *A*). The effect of CT is irreversible even after washing with CT-free solution. This result implies that the inactivation in BTX-modified Na^+ channels is a normal process which also occurs intrinsically in unmodified Na^+ channels and which is not a BTX-induced, novel phenomenon. It is noteworthy that after CT treatment BTX-modified Na^+ currents in general become larger, perhaps because BTX binds preferentially to the open Na^+ channel.

Effect of α-Scorpion Toxin on the Steady-State Inactivation of BTX-modified Na^+ Channels

α-Scorpion toxin is another well-characterized modifier of Na^+ channel inactivation (Catterall, 1980). This class of toxin can slow the inactivation time course of Na^+ channels and render the inactivation incomplete (Wang and Strichartz, 1985). As a result, a maintained current is also evident during a prolonged depolarization.

Like CT, α-scorpion toxin markedly alters the steady-state inactivation curve of BTX-modified Na^+ channels. Fig. 6 *B* (upper trace) shows that inactivation of BTX-modified Na^+ channels was very much reduced in the presence of 200 nM α-scorpion toxin. At this saturating toxin concentration (K_d < 10 nM in the presence of BTX; Catterall, 1980), most of the neuronal Na^+ channels are probably modified by the toxin. The effect of α-scorpion toxin on BTX-modified Na^+ channels is practically irreversible after washing with toxin-free solution. This irreversibility is probably due to a slow dissociation rate of α-scorpion toxin from BTX-modified Na^+ channels. Again, like CT treatment, only ~15% of inactivation occurs at −70 mV (Fig. 6 *B*). Our results thus demonstrate that the inactivation of BTX-modified Na^+ channels is sensitive to both an oxidizing reagent and a peptide toxin that modify the normal inactivation process.

Effect of Benzocaine on Steady-State Inactivation of BTX-modified Na^+ Channels

Benzocaine is a neutral LA that shifts the steady-state inactivation curve of normal Na^+ channels by >20 mV in its hyperpolarizing direction. Benzocaine is thus frequently viewed as an inactivation enhancer because it apparently stabilizes the conformation of the inactivated state (Hille, 1977). Indeed, benzocaine alters the steady-state inactivation curve of BTX-modified Na^+ channels in an opposite manner to that of CT and α-scorpion toxin. Unlike these inactivation modifiers, the effect of benzocaine is completely reversible. Superfusion of GH_3 cells with drug-free solution can readily remove the effect of benzocaine. The steady-state inactivation is drastically enhanced in benzocaine-treated GH_3 cells as compared with the control. In fact, >90% of BTX-activated Na^+ channels are inactivated (and blocked) by benzocaine at −70 mV for 6.5 s vs. ~45% in the absence of benzocaine (Fig. 7, *A* and *B*). Furthermore, inactivation occurs at more negative potentials when benzocaine is present. The midpoint of inactivation, which initially occurred at about −95 mV, is shifted by about −30 mV to −125 mV in the benzocaine-treated GH_3 cells. It is interesting to note here that the steady-state inactivation is also enhanced by benzocaine at voltages larger than −50 mV. However, this induced "inactivation" is continuously relieved as the voltage is increased. It is unlikely that the slowly rising phase of the Na^+ current (Fig. 7 *A*, trace *2*) is due to the rebinding of BTX because all unmodified Na^+ channels are inactivated after 5 ms at +50 mV and therefore cannot bind efficiently with BTX (Khodorov, 1978).

To compare the potency of benzocaine quantitatively at various voltages, we define the apparent binding affinity of benzocaine in BTX-modified Na^+ channels according to the following equation:

$$K_D = [\text{benzocaine}] \cdot I_b / (I_o - I_b)$$

where K_D is the estimated apparent equilibrium dissociation constant, [benzocaine] is 1 mM, and I_b and I_o are the normalized current amplitudes in the presence and absence of benzocaine. Using the mean values of Fig. 7 *B*, we calculated the K_d values for benzocaine at various voltages (Fig. 8). Clearly, benzocaine binding is strongly voltage dependent, ranging from ~8 mM at −170 mV to 0.13 mM at −70 mV. This apparent voltage-dependent binding phenomenon can be directly confirmed by a

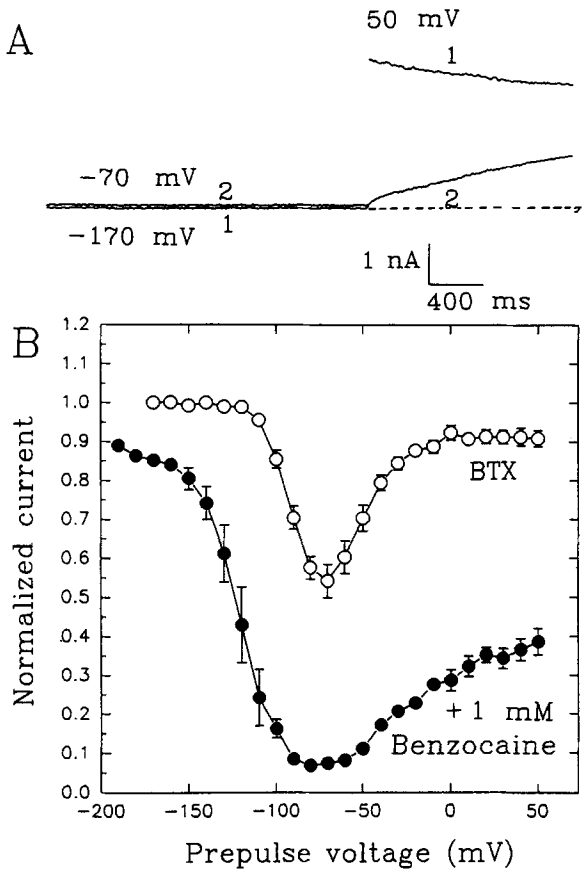


FIGURE 7. Effect of benzocaine on the inactivation of BTX-modified Na^+ channels. (*A*) BTX-modified Na^+ currents at +50 mV were recorded with a prepulse of −170 mV (trace *1*) and −70 mV (trace *2*) after external 1 mM benzocaine treatment for 5 min. Inactivation was clearly enhanced by a −70-mV prepulse with a duration of 6.5 s; <10% of BTX-modified current remained at the first 15 ms of the +50-mV test pulse. (*B*) Steady-state inactivation of BTX-modified Na^+ channels is drastically enhanced by 1 mM benzocaine. Normalized BTX-modified Na^+ currents were plotted against prepulse voltages. The current amplitudes at the first 15–30 ms of a +50-mV test pulse with various prepulse voltages of 6.5 s were measured and normalized against the control current amplitude with a −170-mV prepulse before benzocaine application. Data were pooled from four separate GH_3 cells (*solid circles*), all treated with external 1 mM benzocaine. For comparison, open circles represent the redrawing of Fig. 2 *B* in the absence of benzocaine.

dose–response study at various voltages. These dose–response data will be presented elsewhere.

In contrast to the results described here, Schneider and Dubois (1986) reported that benzocaine binding with BTX-modified Na^+ channels in myelinated nerve fibers is not voltage dependent over a voltage range from −100 to +50 mV. Their findings contrast with Fig. 8, where the binding affinity of benzocaine may differ by a factor of

60. We believe that this difference is mainly due to the pulse protocol used and not due to myelinated nerve fibers per se. In their assay (Schneider and Dubois, 1986), the test pulse duration (without a prepulse) is 20 ms, which is perhaps not long enough for benzocaine binding, as shown in the next section.

Benzocaine Alters Current Kinetics of BTX-modified Na^+ Channels

It is quite evident that the time course of reopening of the inactivated, BTX-modified Na^+ channels is significantly slowed by benzocaine (Fig. 7 *A*). The slowed reopening time course (Fig. 9) can be well fitted with a single exponential, which yields a time constant of 1.35 s. This time constant is ~10 times larger than that without the presence of benzocaine. It is likely that this slowed reopening kinetics is due to the slow dissociation rate of the Na^+ channel-benzocaine complex.

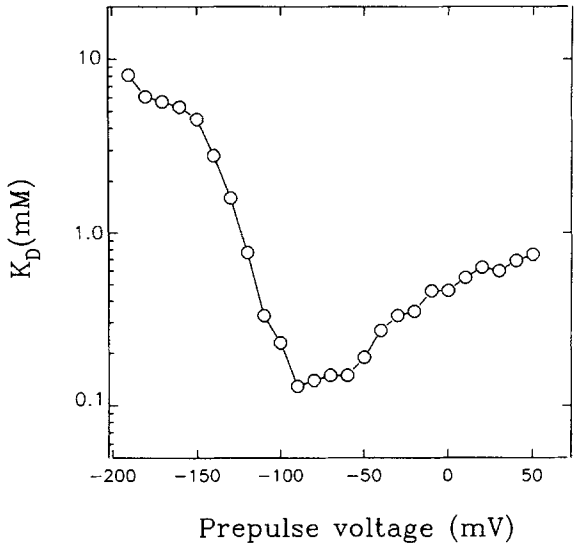


FIGURE 8. Estimated K_D values of benzocaine at various voltages. The K_D value at a given voltage is estimated by an equation:

$$K_D = [\text{benzocaine}] \cdot I_b/(I_o - I_b)$$

where K_D is the estimated dissociation constant, [benzocaine] is 1 mM, and I_b and I_o are the normalized mean current amplitudes in the presence and absence of benzocaine, respectively. Mean I_b and I_o were taken from Fig. 7 *B*.

In addition to the slower reopening time course from inactivation, benzocaine also induces a time-dependent block of BTX-modified Na^+ currents at +50 mV when the resting channels are activated from −170 mV. This time-dependent block can also be described by a single exponential with a time constant of 2.7 s at +50 mV. The degree of this time-dependent block reaches >40% of the peak current amplitude. Together, these results from Figs. 7–9 support the hypothesis that the inactivated state is stabilized by benzocaine and has the highest affinity for benzocaine. Somewhat unexpected, the binding and unbinding of benzocaine with the activator-modified Na^+ channels are relatively slow, in the order of hundreds of milliseconds and seconds. Similar slow binding kinetics of benzocaine have been previously found in muscle BTX-modified Na^+ channels incorporated into planar lipid bilayers (Moczydlowski et al., 1986) and in neuronal veratridine-modified Na^+ channels (Ulbricht and Stoye-Herzog, 1984).

DISCUSSION

Although it is relatively small in size, BTX profoundly alters the normal Na^+ channel function, including the ion selectivity, the gating processes, and the binding of various bioactive ligands such as TTX, α-scorpion toxin, and LAs (Khodorov, 1978; Catterall, 1980; Hille, 1984; Strichartz et al., 1987). It is generally believed that BTX-modified Na^+ channels do not undergo Na^+ channel inactivation (e.g., see Dubois and Coulombe, 1984). In fact, most of the studies in BTX-activated Na^+ channels assume that Na^+ channel inactivation is eliminated by BTX and almost all the kinetic models proposed for BTX-activated Na^+ channels contain no inactivated state (e.g., Tanguy and Yeh, 1988).

Contrary to this general belief, our report indicates that BTX-modified Na^+ channels in GH_3 cells undergo extensive inactivation at voltages near the "resting" potential (i.e., about −70 mV). Approximately 45% of BTX-modified Na^+ channels are inactivated at this potential. This unique phenomenon would have eluded our

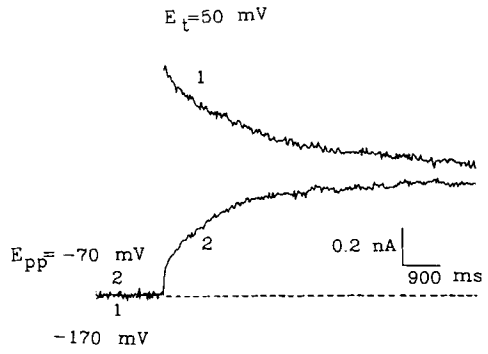


FIGURE 9. Benzocaine binding as revealed by the altered kinetics of BTX-modified Na^+ currents. Benzocaine at 1 mM enhanced the inactivation of BTX-modified Na^+ channels (trace 2, with a −70-mV prepulse for 6.5 s), which reopened ~10 times slower at +50 mV than its normal counterpart without benzocaine. The time course of reopening can be well described by a single exponential function with a time constant of 1.5 s. In addition, benzocaine induced a time-dependent block when channels were activated from their resting state (trace *1*, with a −170-mV prepulse). The time-dependent block can be described by a single exponential with a time constant of 2.26 s. Both curve fittings have R values of ≥ 0.98.

attention if we had not set to study the binding of benzocaine with BTX-modified Na^+ channels.

Steady-State Inactivation of BTX-modified Na^+ Channels

The h_∞ curve of normal Na^+ channels in GH_3 decreases monotonically as the prepulse potential increases from −100 to −20 mV. Nearly all Na^+ channels are inactivated at the prepulse potential of −40 mV. In contrast, the h_∞ of BTX-modified Na^+ channels decreases from −120 mV and reaches its minimal value at −70 mV, where ~45% of BTX-modified Na^+ channels are inactivated. The h_∞ curve then turns upward and reaches a plateau value around 0 mV, which remains constant up to +50 mV. Only ~10% of BTX-modified Na^+ channels are inactivated in this voltage range. There is a considerable overlap of the steady-state inactivation and the activation process of BTX-modified Na^+ channels at voltages between −90 and −40 mV. This result indicates that a significant fraction of Na^+ channels will enter their inactivated state

at −90 to −40 mV, while the resting channels reach their activation threshold and begin to open. The activation time course of BTX-modified Na^+ channels is relatively slow at ≤ −70 mV, as shown in Fig. 1 *B* (see also Huang et al., 1987). On the other hand, the reopening time course from the inactivated BTX-modified Na^+ channels could be relatively fast at −70 mV as extrapolated from Fig. 5 *B*. These two phenomena together could explain the puzzling fact that very little fast inactivating current is present at voltages near −70 mV, despite the fact that 45% of channels are presumably inactivated during a prolonged depolarization. Furthermore, if the activation and inactivation processes overlap extensively as our results indicate, then some of the spontaneous closing events of BTX-activated Na^+ channels in bilayers within this voltage range may be caused by this inactivation process. In fact, there are multiple closed states of BTX-activated Na^+ channels present at voltages near the activation threshold (Moczydlowski et al., 1984). Further experiments are needed to determine whether inactivation is involved in causing some of these closings.

Inactivation Kinetics of BTX-modified Na^+ Channels

Both the development and the recovery of inactivation of BTX-modified Na^+ currents follow second order kinetics. Second order kinetics of inactivation of normal Na^+ channels have been described in myelinated nerve fibers (Chiu, 1977). The fast inactivation time constant of myelinated fibers has a peak value of 30 ms at −80 mV with a bell-shaped voltage dependence similar to that shown in Fig. 5. Chiu (1977) proposed that there are multiple inactivated states present in nerve fibers, $O \rightarrow I_1 \rightarrow I_2$, where O is the open channel and I_1 and I_2 are two inactivated states. It is possible that there is more than one inactivated state of BTX-modified channels, which would account for the fast and slow time constants in the development and recovery of inactivation. However, only one rate-limiting state is required for the reopening of inactivated, BTX-modified Na^+ channels (Fig. 3).

It is also possible that second order kinetics of inactivation may be due to the presence of multiple types of Na^+ channels in GH_3 cells. Schneider and Dubois (1986) studied the effects of benzocaine on Na^+ currents and suggested that there are two types of Na^+ channels in the node of Ranvier, each with different rates of inactivation and different affinities for benzocaine. It is now well established that multiple distinct transcripts of the α subunit of Na^+ channels are present in rat nervous system (Noda et al., 1986). However, it remains to be seen whether rat GH_3 cells express single or multiple types of Na^+ channel transcripts.

In addition, the inactivation process of Na^+ channels may be modulated by small subunits of Na^+ channels or other yet unidentified modulators. Krafte et al. (1988) provided evidence that the products of more than one mRNA species are involved in controlling the inactivation process of rat brain Na^+ channels, whereas Costa et al. (1982) reported that phosphorylation of Na^+ channels can occur, at least in vitro. Both of these modulations may result in producing an additional component of Na current kinetics. Because of these possible explanations to account for the second order kinetics, a unique kinetic scheme of inactivation for the BTX-modified Na^+ channels cannot be ascertained at this time.

Comparison with Previous Results

The steady-state inactivation (h_∞) of ~0.5 of BTX-modified Na^+ channels at about −70 mV has been previously described in the node of Ranvier by Dubois and Coulombe (1984). However, these authors proposed that the Na^+ ion depletion and accumulation in the perinodal space is the cause of this apparent inactivation. A decrease in driving force for Na^+ ions may also cause the Na^+ conductance to decline during a prolonged depolarization.

It is unlikely that Na^+ accumulation and depletion alone can account for our results for the following reasons. First, there is no perinodal space in GH_3 cells to accumulate or deplete the Na^+ ions effectively, as in the node of Ranvier. Furthermore, the external surface of GH_3 was continuously superfused with Na^+-free external solution during our experiments. Second, the maximal inactivation occurs at −70 mV, where only a small BTX-activated Na^+ current is present. If steady-state inactivation is due to Na^+ ion accumulation, the h_∞ curve should continue to decline since outward Na^+ currents and hence external Na^+ ion concentrations become larger at larger depolarizations. We conclude, therefore, that Na^+ ion accumulation at the outer surface of GH_3 cells cannot be a major cause of the inactivation of BTX-modified Na^+ channels in GH_3 cells. It is noteworthy that the slowly declining phase of Na^+ currents in GH_3 cells during a prolonged depolarization (Fig. 1 *B*) may be due in part to the Na^+ ion accumulation in the unstirred layer of the cell membrane. This apparent Na^+ current inactivation, however, can account for no more than 10% of the total outward Na^+ current at +50 mV. Finally, perhaps the strongest evidence for the existence of steady-state inactivation in BTX-modified Na^+ channels comes from the pharmacological studies. Both CT and α-scorpion toxin inhibit the steady-state inactivation, whereas benzocaine enhances it. These results are opposite to the prediction based on the theory of Na^+ ion accumulation and depletion. In the remaining section we will discuss in detail these pharmacological studies.

Does BTX Induce a New Type of Inactivation?

Because BTX alters the Na^+ channel properties extensively, it can be argued that BTX induces a new type of inactivation in GH_3 cells. Our results based on pharmacological studies strongly suggest that this is not the case. Both CT and α-scorpion toxin are established tools for inhibiting the normal Na^+ channel inactivation. It is probably not a fortuitous coincidence that both drugs also alter the steady-state inactivation of BTX-modified Na^+ channels. In addition, benzocaine, a known enhancer of Na^+ channel inactivation, enhances the inactivation of BTX-modified Na^+ channels. Taking these facts together, we conclude that BTX modifies the normal Na^+ channel inactivation and renders it incomplete, which can be further altered by inactivation modifiers such as α-scorpion toxin and CT or inactivation enhancers such as benzocaine.

Tanguy and Yeh (1988) recently reported that the gating charge immobilization can still occur in BTX-modified Na^+ channels as if the "inactivation (h) gate" remains functional, whereas CT effectively removes the charge immobilization. These authors proposed that BTX acts as a ligand that mimics the endogenous h gate and permanently binds with the h gate receptor. In doing so, gating charge immobiliza-

tion will occur normally during depolarization but Na^+ currents will not be blocked by the h gate, presumably because BTX prevents the binding of the normal h gate with its receptor (Tanguy and Yeh, 1988).

An alternative view of the gating charge immobilization in the presence of BTX could also be envisioned after reviewing a model previously provided by Meves (1978). Incomplete inactivation of the Na^+ current was shown to occur in squid axons perfused with NaF. The h_∞ curve rises from a minimum at −20 mV to larger values as the potential becomes more positive (Chandler and Meves, 1970). Meves (1978) hypothesized that the "inactivation gate" which is closed by small depolarizations is forced open again by strong depolarizations. According to this model, the BTX-modified current will be maintained at the large potential since the inactivation gate is forced open by strong depolarization, presumably after charge immobilization. Our results appear to be consistent with Meves's model and suggest that the intrinsic h gate remains functional after BTX treatment. Further gating current experiments are needed to resolve these two possibilities.

State-dependent Block of Benzocaine in BTX-modified Na^+ Channels

What is the underlying mechanism for benzocaine-enhanced inactivation? According to Hille's modulated receptor hypothesis (Hille, 1977), benzocaine has a higher affinity for the inactivated channels than for the resting and open Na^+ channels. As a result, more channels will stay in their inactivated conformation while the receptor is occupied by benzocaine. Our studies on binding interactions between benzocaine and BTX-modified Na^+ channels generally support this hypothesis. Inactivated channels bind most strongly with benzocaine, as revealed by the h_∞ curve of BTX-modified Na^+ channels and the slow time course of reopening of the benzocaine-receptor complex (Figs. 7–9). BTX-modified Na^+ channels in planar bilayers have been reported to be blocked by benzocaine (Moczydlowski et al., 1986). However, unlike cocaine and bupivacaine (Wang, 1988), benzocaine blocks Na^+ channels more efficiently at −50 mV than at +50 mV. This result is now confirmed in GH_3 cells (see Fig. 8). The reason for this apparent voltage-dependent unblock of benzocaine by strong depolarizations is not clear. This phenomenon could be due to conformational changes at the benzocaine binding site of the open channel between −50 and +50 mV. Alternatively, Moczydlowski et al. (1986) suggested that a small fraction of closed Na^+ channels occurs in the voltage range between −50 and +50 mV and such closed conformations of Na^+ channels may be promoted by benzocaine binding. This small fraction of closed Na^+ channels may be in their inactivated state as suggested by the h_∞ curve (Fig. 7) in this voltage range. Finally, our results clearly demonstrate that benzocaine does not bind well with the resting state of BTX-modified channels as compared with the inactivated and perhaps the open counterparts (Figs. 8 and 9). Thus, the modulated receptor hypothesis appears to explain the action of benzocaine on both normal and BTX-modified Na^+ channels. However, BTX reveals an apparent voltage-dependent relief of benzocaine block at strong depolarizations that is not evident in normal Na^+ channels.

We thank Dr. John Daly for providing us with BTX. We are grateful to Dr. Neil Castle for his valuable discussions and to Ms. Rachel Abrams for secretarial services.

This work was supported by grants GM-35401 and GM-39939 from the National Institutes of Health.

Original version received 13 May 1991 and accepted version received 7 October 1991.

REFERENCES

Catterall, W. A. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annual Review of Pharmacology and Toxicology.* 20:15–43.

Chandler, W. H., and H. Meves. 1970. Evidence for two types of sodium conductance in axons perfused with sodium fluoride solution. *Journal of Physiology.* 211:653–678.

Chiu, S. Y. 1977. Inactivation of sodium channels: second order kinetics in myelinated nerve. *Journal of Physiology.* 273:573–596.

Costa, M. R. C., J. E. Casnellie, and W. A. Catterall. 1982. Selective phosphorylation of the α-subunit of the sodium channel by cAMP-dependent protein kinase. *Journal of Biological Chemistry.* 257: 7918–7921.

Cota, G., and C. M. Armstrong. 1989. Sodium channel gating in clonal pituitary cells. The inactivation step is not voltage dependent. *Journal of General Physiology.* 94:213–232.

Dubois, J.-M., and A. Coulombe. 1984. Current-dependent inactivation induced by sodium depletion in normal and batrachotoxin-treated frog node of Ranvier. *Journal of General Physiology.* 84:25–48.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv.* 391:85–100.

Hille, B. 1977. Local anesthetics. Hydrophilic and hydrophobic pathways for the drug receptor reaction. *Journal of General Physiology.* 69:497–515.

Hille, B. 1984. Ionic channels of excitable membrane. Sinauer Associates, Inc., Sunderland, MA. 303–328.

Hodgkin, A. L., and A. F. Huxley. 1952. A quantitative description of membrane current and its application to conduction and excitation in nerve. *Journal of Physiology.* 117:500–544.

Huang, L. Y. M., A. Yatani, and A. M. Brown. 1987. The properties of batrachotoxin-modified cardiac Na channels, including state-dependent block by tetrodotoxin. *Journal of General Physiology.* 90:341–360.

Khodorov, B. I. 1978. Chemicals as tools to study nerve fiber sodium channels: effects of batrachotoxin and some local anesthetics. *In* Membrane Transport Process. Vol. 2. D. C. Tosteson, Yu. A. Ovchinnikov, and Latorre, editors. Raven Press, New York. 153–174.

Krafte, D. S., T. P. Snutch, J. P. Leonard, N. Davidson, and H. A. Lester. 1988. Evidence for the involvement of more than one mRNA species in controlling the inactivation process of rat and rabbit brain Na channels expressed in Xenopus oocytes. *Journal of Neuroscience.* 8:2859–2868.

Krueger, B. K., J. F. Worley, and F. J. French. 1983. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. *Nature.* 303:172–175.

Marquardt, D. W. 1963. An algorithm for least squares estimation of nonlinear parameters. *Journal of Society of Industrial Applied Mathematics.* 11:431.

Meves, H. 1978. Inactivation of the sodium permeability in squid giant nerve fibres. *Progress in Biophysics and Molecular Biology.* 33:207–230.

Moczydlowski, E., S. S. Garber, and C. Miller. 1984. Batrachotoxin-activated Na^+ channels in planar lipid bilayers. Competition of tetrodotoxin block by Na^+. *Journal of General Physiology.* 84:665–686.

Mocyzdlowski, E., A. Uehara, X. Guo, and J. Heiny. 1986. Isochannels and blocking modes of voltage-dependent sodium channels. *Annals of the New York Academy of Sciences.* 479:269–292.

Mozhayeva, G. N., A. P. Naumov, and B. I. Khodorov. 1986. A study of properties of batrachotoxin modified sodium channels. *General Physiology and Biophysics.* 5:17–46.

Noda, M., T. Ikeda, T. Kayano, H. Suzuki, H. Takashima, M. Kurasaki, H. Takahashi, H. Nakayama, and S. Numa. 1986. Existence of distinct sodium channel messenger RNAs in rat brain. *Nature.* 320:188–192.

Rando, T. A., G. K. Wang, and G. R. Strichartz. 1986. The interaction between the activator agents batrachotoxin and veratridine and the gating processes of neuronal sodium channels. *Molecular Pharmacology.* 29:467–477.

Schneider, M. F., and J. M. Dubois. 1986. Effects of benzocaine on the kinetics of normal and batrachotoxin-modified Na channels in frog node of Ranvier. *Biophysical Journal.* 50:523–530.

Strichartz, G. R., T. A. Rando, and G. K. Wang. 1987. An integrated view of the molecular toxinology of sodium channel gating in excitable cells. *Annual Review of Neuroscience.* 10:237–267.

Tanguy, J., and J. Z. Yeh. 1988. Batrachotoxin uncouples gating charge immobilization from fast Na inactivation in squid giant axons. *Biophysical Journal.* 54:719–730.

Ulbricht, W., and M. Stoye-Herzog. 1984. Distinctly different rates of benzocaine action on sodium channels of Ranvier nodes kept open by chloramine-T and veratridine. *Pflügers Archiv.* 402:439–445.

Wang, G. K. 1984. Irreversible modification of sodium channel inactivation in toad myelinated nerve fibers by the oxidant chloramine-T. *Journal of Physiology.* 346:127–141.

Wang, G. K. 1988. Cocaine-induced closures of single batrachotoxin-activated Na^+ channels in planar lipid bilayers. *Journal of General Physiology.* 92:747–765.

Wang, G. K. 1990. Binding affinity and stereoselectivity of local anesthetics in single batrachotoxin-activated Na^+ channels. *Journal of General Physiology.* 96:1105–1127.

Wang, G. K., and G. R. Strichartz. 1983. Purification and physiological characterization of neurotoxins from venoms of the scorpions *Centruroides sculpturatus and Leiurus quinquestriatus. Molecular Pharmacology.* 23:519–533.

Wang, G. K., and G. R. Strichartz. 1985. Kinetic analysis of the action of *Leiurus* scorpion α-toxin on ionic currents in myelinated nerve. *Journal of General Physiology.* 86:739–762.

Zubov, A. N., A. P. Naumov, and B. I. Khodorov. 1984. Effects of batrachotoxin on sodium channels of neuroblastoma cells. *Neirofiziologiya.* 16:415–423.