BATRACHOTOXIN UNCOUPLES GATING CHARGE IMMOBILIZATION FROM FAST Na INACTIVATION IN SQUID GIANT AXONS

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ABSTRACT The fast inactivation of sodium currents and the immobilization of sodium gating charge are thought to be closely coupled to each other. This notion was tested in the squid axon in which kinetics and steady-state properties of the gating charge movement were compared before and after removal of the Na inactivation by batrachotoxin (BTX), pronase, or chloramine-T. The immobilization of gating charge was determined by measuring the total charge movement (Q_{ON}) obtained by integrating the ON gating current ($I_{g,ON}$) using a double pulse protocol. After removal of the fast inactivation with pronase or chloramine-T, the gating charge movement was no longer immobilized. In contrast, after BTX modification, the channels still exhibited an immobilization of the gating charge (Q_{ON}) with an onset time course and voltage dependence similar to that for the activation process. These results show that BTX can uncouple the charge immobilization from the fast Na inactivation mechanism, suggesting that the Na gating charge movement can be immobilized independently of the inactivation of the channel.

INTRODUCTION

During a maintained membrane depolarization, the Na channel is inactivated (Hodgkin and Huxley, 1952), and the gating charge is partially immobilized (Bezanilla and Armstrong, 1974; Meves and Vogel, 1977; Nonner et al., 1975; Nonner, 1980). The relationship between these two events remains controversial. Armstrong and Bezanilla (1977) first described them as being closely linked together. Consistent with this notion is the observation that these two processes occur with similar time course and voltage dependence. In addition, pronase treatment, which removes the Na current inactivation, abolishes the gating charge immobilization. Nonner (1980) proposed that gating charge immobilization is linked to some steps of a high-order inactivation process and Greeff et al. (1982) attributed the inactivating and noninactivating components to different origins. The fast inactivation in squid axons can be removed by several agents including pronase (Armstrong et al., 1973; Rojas and Rudy, 1976), N-bromoacetamide (Oxford et al., 1978; Oxford, 1981), chloramine-T (Wang et al., 1985; Huang et al., 1987), and batrachotoxin (BTX) (Tanguy et al., 1984). Chloramine-T is thought to act differently from pronase (Wang et al., 1985; Rack et al., 1986). From Na current experiments in squid axon, we have shown that BTX removes the fast inactivation differently from pronase (Tanguy et al., 1984). Thus, these agents may serve as useful tools to probe the link between fast inactivation and gating charge immobilization. We present in this paper a parallel study of the effects of BTX on the fast Na inactivation and on the kinetics and voltage-dependence of the gating charge immobilization by the ON gating current measurement, and a comparative study of the effects of BTX, chloramine-T, and pronase on the gating charge immobilization. We demonstrate that chloramine-T suppresses the gating charge immobilization similarly to pronase, whereas BTX removes Na inactivation without suppressing the charge immobilization. The mechanism by which BTX could uncouple the fast inactivation from the charge immobilization is discussed. A preliminary report of this study has been presented (Tanguy et al., 1987).

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METHODS

Experiments were performed on giant axons isolated from squid, *Loligo pealei*, obtained at the Marine Biological Laboratory (Woods Hole, MA). Axons were internally perfused by the roller method (Baker et al., 1961) and voltage clamped with the axial wire electrode technique as previously described (Oxford, 1981). 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. Feedback circuit for the series resistance compensation was used in all Na current experiments and in some of gating current experiments to compensate for errors arising from series resistance. For Na current measurements, the axon was perfused externally with artificial seawater containing (in mM): Na^+, 450; Ca^{++}, 50; Cl^-, 550; and Hepes, 10 (pH 7.3) and in some experiments 7 nM tetrodotoxin (TTX) added to reduce the amplitude of Na current. The internal solution contained (in mM): Na^+, 300; glutamate$^-$, 220; F^-, 50; sucrose, 400; phosphate buffer, 15 (pH 7.3). For the gating current measurements, the axon was perfused with an internal solution containing (in mM): Cs^+,

300; F^-, 100; glutamate$^-$, 200; sucrose, 400; MOPS buffer, 10 (pH 7.3) and superfused with an external solution containing (in mM): TMA^+ (tetramethylammonium), 450; Ca^{++}, 50; Cl^-, 550; Hepes buffer, 10 (pH 7.3); and 2 μM TTX.

Removal of the fast inactivation of the Na current was carried out with three agents internally applied: BTX, chloramine-T, and pronase. In all the experiments with BTX, a repetitive stimulation (a train of 4-ms depolarizing pulses from −80 mV to +80 mV at 10 Hz) was applied for ~10 min at the beginning of the internal perfusion of BTX (4 μM) to induce a complete modification of the Na currents; namely, a complete removal of the fast Na inactivation and the activation of the Na conductance at very negative potentials. Once the channels were modified, the internal solution was switched to BTX-free internal solution. The modification was found to persist for the entire period of experiment (up to 4 h). Thus, the Na channels were irreversibly modified by BTX. In the gating current experiments, this protocol was applied before the application of TTX, so that the completeness of the BTX modification could be established. The persistence of removal of the Na inactivation by BTX in the presence of TTX was verified by washing out TTX at the end of the gating current experiment (see Fig. 6). In all experiments with chloramine-T, a freshly prepared solution containing 7.5 mM chloramine-T was internally applied for ~10 min to irreversibly remove the inactivation of Na current (Huang et al., 1987). Similarly, a freshly prepared pronase solution at 0.2 mg/ml was applied internally to the axon for ~15 min at 10°C. BTX was a generous gift of Dr. John W. Daly from National Institutes of Health, Chloramine-T (*N*-chloro-*p*-toluenesulfonamide sodium salt) was purchased from Sigma Chemical Co. (St. Louis, MO). Pronase (from *Streptomyces griseus*) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). All experiments were performed at 9–10°C.

The voltage clamp step was generated from a computer PDP 11/73 (Digital Equipment Corp., Marlboro, MA), and membrane currents were sampled at 10 μs per point 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 for Na current measurements and with the −P/4 pulse starting from the potential of −150 mV for gating current measurements. The charge immobilization was studied with the double-pulse protocol in which P − P/4 method was used for both pulses (Armstrong and Bezanilla, 1977). The total gating charge (Q_{ON}) was obtained by integrating the ON gating current ($I_{g,ON}$) for ~2 ms. To determine the time constants of the exponential time courses, the data points were fitted by a least-square algorithm for simultaneous multiexponential fit. Average values of parameters in the text are expressed as means ± SD.

The following kinetic model previously presented by Armstrong and Gilly (1979) was used for simulating the Na currents and the onset of charge immobilization before and after BTX treatment

$$\begin{array}{ccccccccccc} & & & & & & & & \mathrm{CI} & \underset{\beta_6}{\overset{\alpha_6}{\rightleftharpoons}} & \mathrm{I} \\ & & & & & & & & l' \Big\| k' & & l \Big\| k \\ \mathrm{C_1} & \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} & \mathrm{C_2} & \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} & \mathrm{C_3} & \underset{\beta_3}{\overset{\alpha_3}{\rightleftharpoons}} & \mathrm{C_4} & \underset{\beta_4}{\overset{\alpha_4}{\rightleftharpoons}} & \mathrm{C_5} & \underset{\beta_5}{\overset{\alpha_5}{\rightleftharpoons}} & \mathrm{O}\ , \end{array}$$

Scheme I

where C_1 to C_5 represent the closed states of the Na channels, O the open state, I the inactivated state, and CI the closed inactivated state. The kinetic model was numerically solved using the 4th and 5th order of the Runge-Kutta method. To generate the Na currents at +20 mV, it was assumed as the initial condition that all channels are in state C_1 at rest (at −80 mV). After prepulses, the initial condition changed according to the calculated distribution of the channels at the end of the interpulse as defined in the double-pulse protocol used in the experiments. The Na current proportional to the conductance of the conducting states, was calculated by numerically integrating the eight first-order differential equations describing the scheme above. In the control, the Na current represents the product of the driving force ($E_m - E_{Na}$) and the conductance of the conducting state O ($\gamma_{(O)}$). In the BTX-modified channels, since state I is transformed to the conducting state O*, the Na current represents the product of the same driving force as in control and the sum of the conductances of the two open states ($\gamma_{(O)} + \gamma_{(O^*)}$), assuming that the single conductance in O was the same as in the control, and that the single conductance in O* was 70% of that in state O.

The gating currents were calculated assuming the same relations and assumptions for determining the rate constants as Armstrong and Gilly

TABLE I
PARAMETERS FOR CALCULATION

Rate constants	−80 mV		20 mV		Transition	Q	
	Control	BTX	Control	BTX		Control	BTX
	ms^{-1}	ms^{-1}	ms^{-1}	ms^{-1}			
Alpha 1	5.87	5.87	10.8	10.8	1	1	1
Alpha 2	7.6	7.6	14	14	2	1	1
Alpha 3	7.6	7.6	14	14	3	1	1
Alpha 4	7.6	7.6	14	14	4	1	1
Alpha 5	0	0	5	5	5	2	2
Alpha 6	0	0	5	5	6	2	2
Beta 1	30.9	30.9	2.33	2.33			
Beta 2	40	40	3.01	3.01			
Beta 3	40	40	3.01	3.01			
Beta 4	40	40	3.01	3.01			
Beta 5	10	1	0.089	0.089			
Beta 6	10	5	0.089	0.089			
k	0	0	1	3	O ↔ I	0	0
l	0	0	0.1	0.5			
k'	0	0	0.1	0.02	$C_5 \leftrightarrow C_1I$	0	0
l'	1	1	0.01	0.003			

(1979). The following equation was used to calculate the gating current associated for each step.

$$I_{g,(C_n \leftrightarrow C_{n+1})} = Q_n(dx_{n+1}/dt) = Q_n(\alpha_n x_n - \beta_n x_{n+1}),$$

where x_n represents the fraction of the channels in the state C_n, Q_n the gating charge associated with the transition $C_n \leftrightarrow C_{n+1}$, and α_n and β_n the forward and backward rate constant of the transition $C_n \leftrightarrow C_{n+1}$. The total gating current $I_{g,ON}$ was obtained by summing the I_g calculated for each step. The parameters used for calculating the rate constants were adjusted to fit satisfactorily the kinetics of the Na inactivation and gating charge immobilization in control. These parameters were then empirically modified a minima from the original model to fit satisfactorily the essential characteristics of the Na currents and gating charge movement of the BTX-modified channels. Table I gives the rate constants and the gating charge associated with each transition for normal and BTX-modified channels, according to Scheme I.

Values for the rate constants are given in ms^{-1}. The transition n ($\eta \leq 5$) represents the transition between C_n and C_{n+1}, and the transition 6 the transition between CI and I in the control or between CI and O* in the BTX-modified channels. The gating charge Q_n associated with the transition n is expressed as the number of electronic charge e, assuming that the gating charges move through the total electrical distance ($\delta = 1$).

RESULTS

BTX Removes the Fast Na Inactivation

The fast Na inactivation in squid axons followed a single exponential time course, which can be studied by three methods using either a single-pulse or a double-pulse protocol. In Fig. 1, the time constant of inactivation and the degree of removal of inactivation by BTX were determined before and after BTX-treatment by three different methods. (*a*) The time constant (τ_h) determined from the decay phase of the Na current during a single depolarization step (Fig. 1, *A* and *B*) was 1.02 ms at +20mV in the

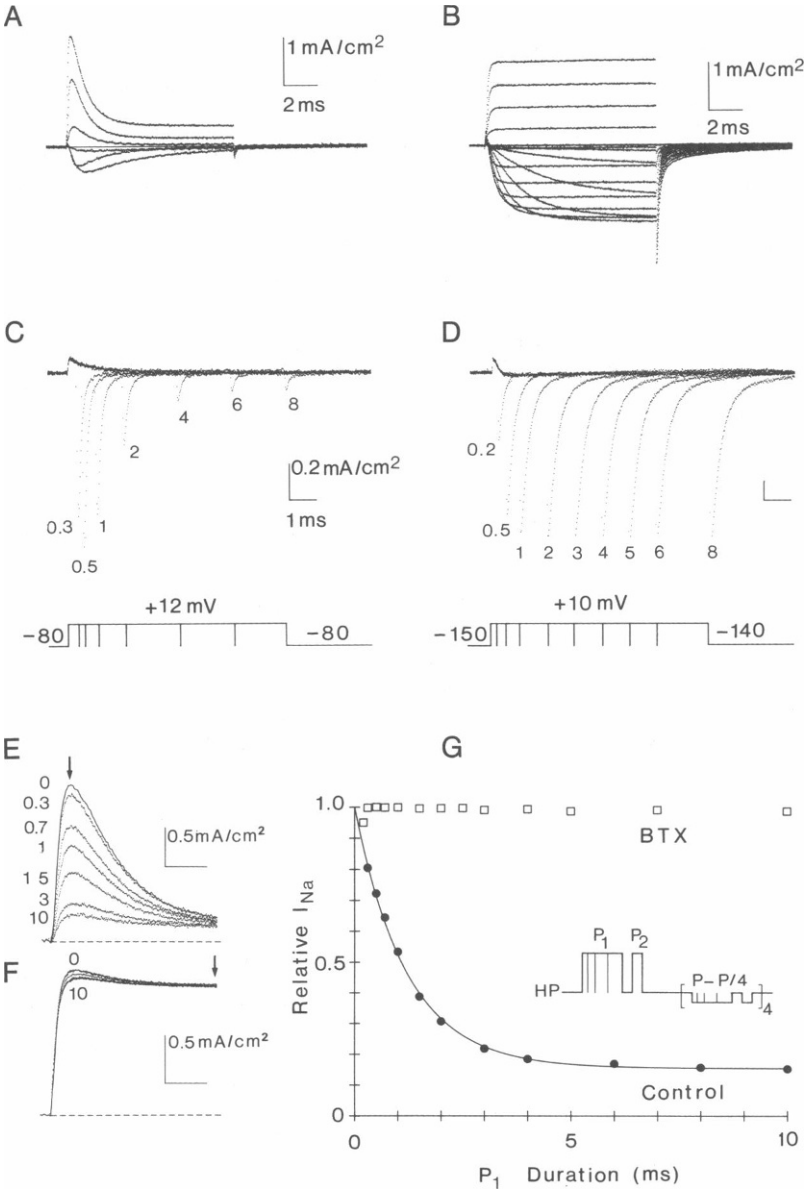

FIGURE 1 BTX removed the fast Na inactivation. The fast inactivation was determined before (*A*, *C*, and *E*) and after BTX treatment (*B*, *D*, and *F*) by three methods: the decaying phase of Na current during depolarization (*A* and *B*), the Na tail current method (*C* and *D*) and the double-pulse protocol (*E*, *F*, and *G*). The families of Na currents were recorded during 10-ms step depolarizations from −40 to +60 mV (by 20-mV increment) applied from a holding potential (HP) of −80 mV in control (*A*), and from −100 to +50 mV (by 10-mV increment) after BTX treatment (HP = −150 mV) (*B*). As illustrated in *B*, the decaying phase of the sodium current was irreversibly abolished by the internal application of 4μM BTX after 6,400 pulses (each of 4 ms in duration) applied from −80 mV to +80 mV at 10 Hz. In *C*, the decrease of the amplitude of the instantaneous current as a function of the pulse duration followed a single exponential time course with a time constant of 1.44 ms, reflecting the fast inactivation kinetics at +12 mV. After BTX treatment (*D*), no decay of the amplitude of the instantaneous current was observed. In *E*, according to the protocol in *G*, the control Na currents were recorded at +20 mV in response to the test pulse (P_2) for the following durations of the conditioning pulse (P_1) to +20 mV of 0, 0.3, 0.7, 1, 1.5, 3, and 10 ms (as labeled from the top to bottom trace successively). In *F*, the Na currents were recorded after nearly complete BTX treatment at P_2 = +20 mV after a conditioning pulse to E_{Na} (P = +10 mV) for 0, 0.3, 1, 3, 5, and 10 ms. The amplitude of the peak Na current in *E* and of the steady-state current in *F* normalized to their respective values measured in the absence of P_1 were plotted as a function of P_1 duration in *G*. The control peak current decayed monoexponentially with a time constant of 1.21 ms, whereas the steady-state current after BTX treatment was not affected at all by P_1.

control (Fig. 1 *A*), whereas after BTX treatment there was no decrease in the Na current amplitude during a 10-ms depolarizing pulse at all potentials (Fig. 1 *B*). (*b*) The decrease of the instantaneous current upon repolarization after various durations of the depolarizing pulse (according to the protocol of Fig. 1 *C*) had a similar time constant as the decaying phase of the Na current in the control (τ_h was 1.44 ms at 12 mV in Fig. 1 *C*). After BTX treatment, no decrease in this instantaneous current was observed. (*c*) In the double-pulse method (see the inset of Fig. 1 *G*), the decrease of the peak Na current elicited by the test pulse (P_2) with the lengthening of the conditioning pulse (P_1) was used to determine τ_h (Fig. 1, *E–G*). In the control, τ_h was 1.21 ms at +20 mV (Fig. 1 *G*). In addition, Fig. 1 *F* and *G* shows that the double-pulse protocol is a sensitive method to determine the degree of removal of inactivation: the steady-state current was not reduced by the conditioning pulse, whereas a very small decrease of the initial peak current still followed a similar time course as the control, indicating that, in this axon, 95% of the Na channels were modified. In all experiments presented in this paper, more than 95% of the channels were modified, as estimated from the degree of removal of inactivation. These results clearly show that the inactivation of the Na current has been removed by BTX treatment. For the sake of completeness, Fig. 1 *B* also illustrates the other action of BTX, namely, to open Na channels at very negative potentials, which is reflected in the hyperpolarization shift of voltage dependence of conductance increase, as observed in other preparations (for review, see Khodorov, 1985). In this axon, for example, the voltage corresponding to half of the maximum conductance increase was shifted by 54 mV from −12 to −66 mV after BTX modification. However, the present paper will mainly address BTX action on the Na inactivation mechanism.

Onset Time Course of Gating Charge Immobilization before and after BTX Treatment

The gating charge immobilization can be measured directly from the OFF gating current ($I_{g,OFF}$) after a single depolarizing pulse, or from the ON gating current ($I_{g,ON}$) by a double-pulse protocol. The double-pulse protocol was used to study the kinetics and steady-state properties of charge immobilization. As shown in the inset of Fig. 2 *D*, the ON gating current ($I_{g,ON}$) associated with a test pulse (P_2) to +20 mV was used to measure the gating charge which was still able to move after various durations of the conditioning pulse (P_1). Selected traces of gating current $I_{g,ON}$ associated with P_2 following the indicated durations of P_1 are shown in Fig. 2, *A* and *C*. The total Q_{ON} associated with P_2 (see Methods) decreased with the P_1 duration, following a time course which was determined after normalizing Q_{ON} to its initial value (Fig. 2, *B* and *D*). In the control (Fig. 2 *B*), the decrease of Q_{ON} followed a single exponential time course with time constant of 1.0 ± 0.3 ms ($n = 4$) reaching a steady-state value of 0.43 ± 0.12 ($n = 4$). This time constant is comparable to the time constant of fast Na inactivation. The steady-state value

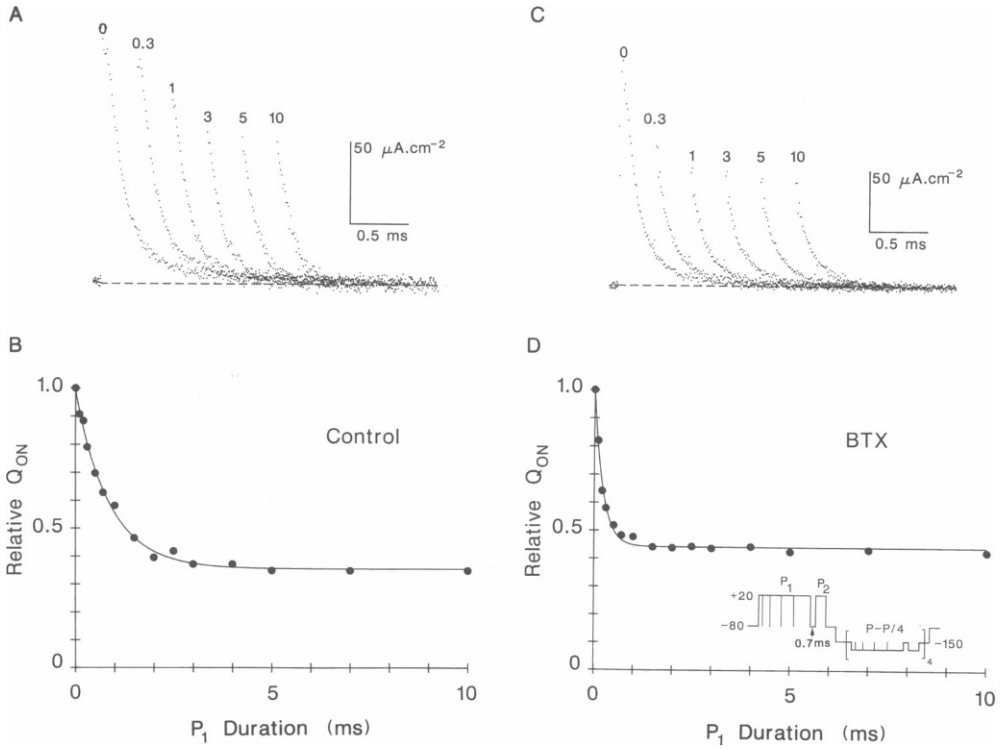

FIGURE 2 The gating charge immobilization was not removed by BTX. The gating charge immobilization was measured by the double-pulse protocol (each pulse with $P - P/4$) illustrated in the inset of *D*. In *A* (control) and *C* (BTX-treated axon), the gating currents ($I_{g,ON}$) associated with P_2 are displayed with indicated duration (in ms) of conditioning pulse (P_1). In *B* and *D*, the Q_{ON}, obtained by integrating $I_{g,ON}$ from 0 to ~2 ms, was normalized to its initial value. The decrease of Q_{ON} followed a single exponential time course with a time constant of 0.86 ms in the control (*B*) and 0.22 ms in the BTX-treated axon (*D*), and the steady-state values, which represent the immobilization-resistant component were 0.40 and 0.45 before and after BTX modification, respectively.

estimated by the double-pulse protocol represents the immobilization-resistant component of the charge movement, which also appears as the steady-state fraction of the fast component of $I_{g,OFF}$. However, the extent of the charge immobilization, as determined by both methods, is less complete than that of the Na inactivation, as previously reported (Armstrong and Bezanilla, 1977; Meves and Vogel, 1977; Nonner, 1980).

After BTX modification, the total Q_{ON} decreased as the P_1 duration was lengthened despite the fact that the inactivation of Na current had been eliminated. After various durations of P_1 to +20 mV, the decrease in Q_{ON} still followed a single exponential time course with a time constant of 0.35 ± 0.14 ms (n = 5), and reached a steady-state value of 0.42 ± 0.07 (n = 5). Thus, the onset of charge immobilization becomes approximately three times faster in the BTX-modified axon than in an axon with intact inactivation. The difference in the steady-state value for the immobilization-resistant component is not statistically significant.

The degree of the charge immobilization could also be detected by measuring $I_{g,OFF}$. Because of a large variability in the slow component of the $I_{g,OFF}$, there was an uncertainty in estimating the total charge (Q_{OFF}) corresponding to $I_{g,OFF}$ by a direct integration method (Nonner, 1980). One way to overcome this problem is to decompose the $I_{g,OFF}$ into a fast and a slow component. By measuring the decrease of the fast component of $I_{g,OFF}$ at −80 mV as a function of pulse duration, one can determine the onset and degree of charge immobilization. In the control, the gating charge immobilization at +20 mV occurred with a time constant of 1.16 ± 0.29 ms and with a noninactivating component of 28% ± 10% (n = 5). Thus, the time constant determined by the $I_{g,OFF}$ method was similar to that determined by the double-pulse protocol. In BTX-treated axon, when the $I_{g,OFF}$, after a 4-ms depolarizing pulse to +20 mV, was integrated at −80 or at −70 mV, the total Q_{OFF} was found to be 56 and 35%, respectively, of that obtained from $I_{g,OFF}$ at −150 mV. Thus, by the $I_{g,OFF}$ measurement ~50% of the gating charge became immobilized by depolarizing to +20 mV. However, the measurement of $I_{g,OFF}$ could not reliably detect the time course of charge immobilization of the BTX-modified channels for the following two reasons. First, the charge immobilization occurred so rapidly (as estimated from the double-pulse protocol) that its onset could not be monitored by direct measurement of $I_{g,OFF}$. Second, the kinetics of $I_{g,OFF}$ was slowed down as compared with the control, by mostly increasing the fast time constant. As a result, the kinetic analysis involving the decomposition of the $I_{g,OFF}$ into the fast and slow components became less reliable to estimate the fast component of the total Q_{OFF}.

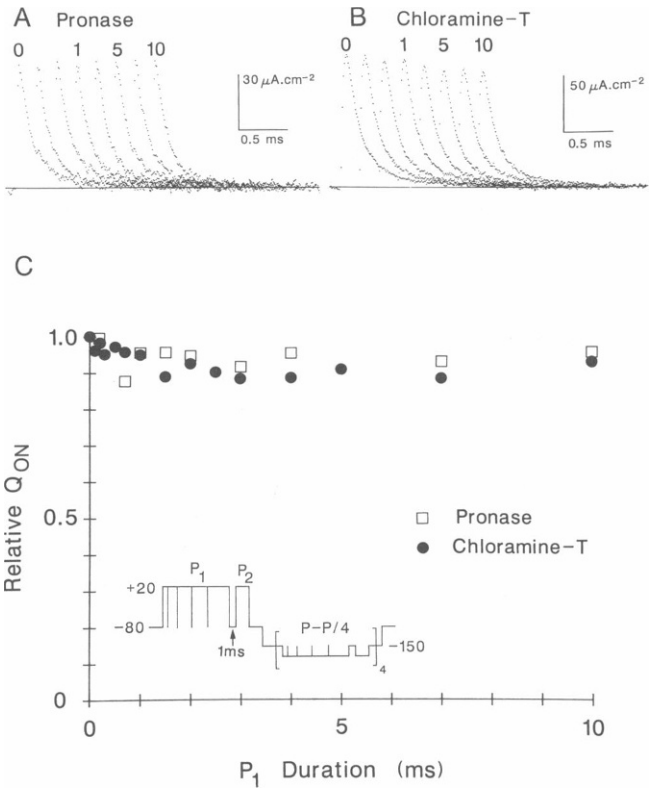

FIGURE 3 Gating charge immobilization was abolished by pronase and chloramine-T treatment. The gating charge immobilization was determined after pronase and chloramine-T treatment by the double-pulse protocol shown in *C*. In *A* and *B*, the $I_{g,ON}$ traces associated with the test pulse P_2 are displayed for various P_1 durations (0, 0.3, 0.7, 1, 3, 5, 7, and 10 ms after pronase in *A*, and 0, 0.3, 0.7, 1, 3, 5, 7, and 10 ms after chloramine-T in *B*, as indicated for some of the traces). The Q_{ON} associated with P_2 was normalized to its initial value and plotted as a function of P_1 duration in *C*. No significant decrease of Q_{ON} was observed after pronase (*open squares*) or chloramine-T (*solid circles*) treatment.

Chloramine-T and Pronase Suppress the Gating Charge Immobilization

After pronase treatment, there was no decrease in $I_{g,ON}$ when the P_1 duration was lengthened (Figs. 3, *A* and *C*). Thus, charge immobilization is removed by pronase treatment, as previously shown by Armstrong and Bezanilla (1977) using the measurement of $I_{g,OFF}$. In addition, we demonstrated here that no decrease of $I_{g,ON}$ with P_1 duration was observed after chloramine-T treatment (Fig. 3, *B* and *C*). Thus, similar to pronase, chloramine-T, which removes the Na inactivation (Wang et al., 1985; Huang et al., 1987), suppressed the gating charge immobilization in squid axons.

Voltage-dependence of Gating Charge Immobilization before and after BTX Treatment

Inasmuch as the removal of inactivation by BTX was not associated with any loss of charge immobilization, it was interesting to compare the voltage-dependence of the charge immobilization before and after BTX treatment. To obtain these results, the double-pulse protocol, shown in the inset of Fig. 4 *B*, was used before and after removal of

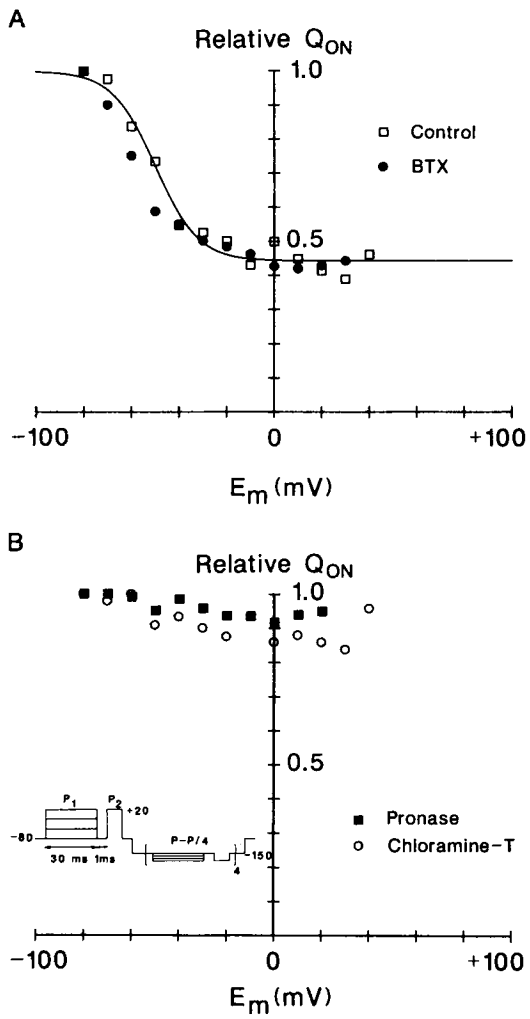

FIGURE 4 The voltage-dependence of gating charge immobilization was slightly shifted by BTX modification. The voltage-dependence of the charge immobilization was determined by the double-pulse protocol shown in the inset of *B* after the removal of the fast inactivation by BTX (*A*) or by pronase or chloramine-T (*B*). In *A* and *B*, the total charge movement (Q_{ON}) was normalized to its value measured for $P_1 = -80$ mV and plotted as a function of P_1 amplitude. In *A*, Q_{ON} decreased as the amplitude of P_1 increased from −80 to +40 mV, in control (*open squares*) and after BTX treatment (*solid circles*). The control values have been fit by a modified Boltzmann equation $Y(t) = ((1 - B)/\{1 + \exp[(E - E_{1/2})/S]\}) + B$, with $E_{1/2} = -50$ mV, $B = 0.44$, and $S = 9.0$ mV. After BTX treatment, $E_{1/2}$ was slightly shifted towards the hyperpolarizing direction ($E_{1/2} = -57.4$ mV) but the steady-state value ($B = 0.45$) and the slope factor ($S = 8.8$ mV) were not significantly different from the control. In *B*, Q_{ON} was only slightly decreased by P_1 after pronase (*solid squares*) or chloramine-T treatment (*open circles*).

inactivation by BTX (Fig. 4 *A*). This protocol was repeated with amplitudes of P_1 varying from −80 to +40 mV. The total Q_{ON} associated with test pulse P_2 decreased as P_1 amplitude was increased both in the control and after BTX treatment. The voltage dependence of charge immobilization could be described in either case by a modified Boltzmann equation defined by three parameters: (*a*) The midpoint potential for the decrease in total Q_{ON}, which was shifted towards the hyperpolarizing direction from −48 ± 3 mV ($n = 2$) in the control to −58 ± 5 mV ($n = 7$) after BTX modification, (*b*) the steady-state level of immobilization, which was 0.35 ± 0.12 ($n = 2$) and 0.51 ± 0.10 ($n = 7$), for control and BTX-modified channels, respectively, and (*c*) the slope factor (per an *e*-fold decrease in charge movement), which was 10.5 ± 2.1 mV ($n = 2$) and 8.2 ± 1.3 mV ($n = 7$), for the control and the BTX-modified channels, respectively. Thus, the voltage dependence of charge immobilization was shifted on average by 10 mV towards hyperpolarizing direction after BTX modification. As expected from the suppression of gating charge immobilization, the gating charge movement was no longer decreased by depolarizing conditioning potentials after treatment with pronase or chloramine-T, as illustrated in Fig. 4 *B*.

The Q_{ON}–V Relation before and after BTX Modification

As shown in Fig. 5, the voltage-dependence of the total gating charge movement (Q_{ON}–V curve) was not significantly altered after BTX modification. We further compare the *h*-gate–induced and BTX-induced charge immobilization by investigating the effect of a conditioning depolarizing pulse on the Q_{ON}–V curve. In the control axon, a conditioning potential which causes inactivation of the Na currents and immobilization of the gating charge

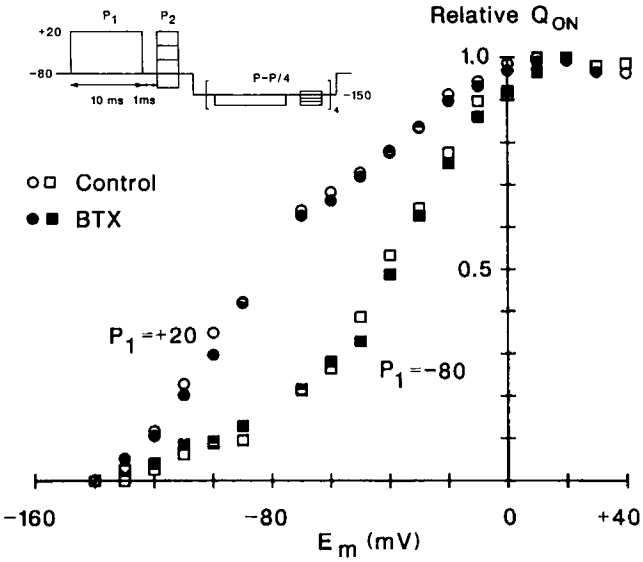

FIGURE 5 The Q_{ON}–V distribution was shifted by depolarizing pulse in the control and BTX-modified channels. The Q_{ON}–V distribution with or without prior conditioning pulse to +20 mV was determined by the double-pulse protocol shown in the inset of the figure. The ON gating current associated with P_2 was integrated for 2 ms to obtain the total Q_{ON}. To facilitate the comparison, the absolute value of the most negative Q_{ON} was added to all other data points and normalized to the maximal value and was plotted as the function of P_2 (designated as E_m). In the control axon, the Q_{ON}–V curve with a conditioning pulse ($P_1 = +20$ mV) (○) lies about 40 mV left to the one without conditioning pulse ($P_1 = -80$ mV) (□). After BTX modification, both Q_{ON}–V curves with or without +20 mV conditioning pulse almost superimposed on the control ones, respectively.

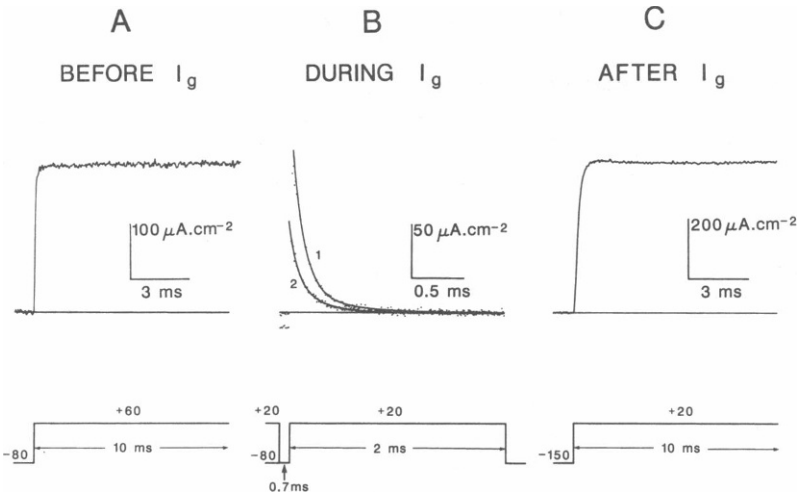

FIGURE 6 TTX did not displace BTX from its binding site. After BTX treatment, the Na currents were measured before (*A*) and after (*C*) the determination of the gating charge immobilization onset which was performed under external application of TTX (*B*). In *A*, the current was recorded at +60 mV in 300 Cs, K-free internal solution and Na-free, K-free external solution according to the pulse protocol represented below the current trace. The rising phase of the current trace represents the gating current and the steady-state current is Na current, which failed to inactivate indicating a complete removal of the fast Na inactivation after BTX treatment. After external application of 2 μM TTX, the Na currents were completely abolished and the ON gating currents were recorded with and without a conditioning pulse to determine the gating charge immobilization. In *B*, the $I_{g,ON}$ traces (traces *1* and *2*) associated with the test pulse P_2 for two different P_1 durations (0 and 7 ms, respectively), according to the pulse protocol shown below, are superimposed. The decay phase of both traces was well described by a sum of two exponential functions of time of the form: $A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3$, where τ_1 and τ_2 are the fast and slow time constants respectively, and A_1, A_2, and A_3 the coefficients proportional to the initial amplitudes of the fast and slow components, and to the steady-state component, respectively. Exponential fits are shown by the continuous lines drawn through the data points. In the absence of P_1 (trace *1*) the $I_{g,ON}$ decayed with τ_1 = 107 μs (A_1 = 255 μA · cm^{-2}), τ_2 = 404 μs (A_2 = 33 μA · cm^{-2}). After the 7-ms conditioning pulse to +20 mV (trace *2*), the $I_{g,ON}$ kinetics became faster with τ_1 = 77 μs (A_1 = 72 μA · cm^{-2}) and τ_2 = 243 μs (A_2 = 46 μA · cm^{-2}) and the total Q_{ON} decreased to ~40% of its value obtained in absence of P_1 (trace *1*), indicating that the gating charge was immobilized by the depolarizing prepulse. After washing away TTX, the Na currents were recorded in 300 Na, K-free internal and external media, as shown in *C*. The Na current trace at +20 mV (see pulse protocol), which was obtained by the TTX subtraction method, failed to inactivate, indicating a persistent removal of the fast Na inactivation induced by BTX.

would shift the Q_{ON}–V relationship to more negative potentials (Armstrong and Bezanilla, 1977; Bezanilla et al., 1982). Fig. 5 shows that a 10-ms depolarizing pulse to +20 mV caused a shift of about 40 mV of the Q_{ON}–V curve to the hyperpolarizing direction. After BTX modification, the Q_{ON}–V curve with a conditioning pulse to +20 mV resembled the control one. The results clearly demonstrate two important aspects of BTX modification of the Na channels in squid axons. First, the Q_{ON}–V curve is not shifted appreciably by BTX modification despite the fact that the voltage-dependence of the conductance (g–V curve) has been shifted by ~50 mV in the hyperpolarizing direction. Second, the conditioning potential to +20 mV could still shift the Q_{ON}–V curve as seen in the control axon despite the fact that the Na current inactivation has been removed.

Kinetics of the ON Gating Current before and after BTX Modification

The analysis of the ON gating current kinetics failed to reveal marked changes in the BTX-modified channels (see records in Fig. 2, *A* and *C*). In the control, the decay of $I_{g,ON}$ followed a double-exponential kinetics with a fast time constant (τ_f) of 93 ± 19 μs (n = 6) (for 53.2 ± 13.4% of the total Q_{ON}) and a slow time constant (τ_s) of 440 ± 95 μs (n = 6). After BTX modification, $I_{g,ON}$ decayed also biexponentially with τ_f = 102 ± 18 μs (n = 4) (for 63 ± 4% of the total Q_{ON}) and with τ_s = 358 ± 61 μs (n = 4). Likewise, kinetic changes of the decay of $I_{g,ON}$ with depolarizing prepulses were similar in the control and in the BTX-modified channel. As previously described in axon with intact inactivation by Armstrong and Gilly (1979), a depolarizing prepulse producing Na inactivation reduces the total charge movement and speeds up the decay of $I_{g,ON}$. After BTX treatment, which completely removed Na current inactivation (Fig. 6, *A* and *C*), a prepulse to +20 mV applied for 10 ms clearly decreased the total charge movement and accelerated the decay of $I_{g,ON}$, as illustrated in Fig. 6 *B*. The decaying phase of the inactivation-resistant charge movement still followed a double-exponential time course. As in intact axons, the prepulse induced an acceleration in $I_{g,ON}$ either by decreasing the proportion of the slow component without much effect on the time constant, or by increasing the fast time constant without changing much their proportions, as in Fig. 6 *B*.

Gating Charge Immobilization Observed after BTX Treatment Is Not Associated with Unmodified Na Channels

All these effects of BTX on the gating currents were observed after a complete removal of Na inactivation, as illustrated in Fig. 6, *A* and *B*. This result makes unlikely the explanation that the gating charge immobilization observed after BTX treatment is due to unmodified Na channels, as recently proposed by Meves et al. (1987). In the Na current experiment, the BTX-induced modification was essentially irreversible even after having perfused the axon with a BTX-free solution for as long as 4 h. In the gating current experiment, however, one could imagine that the BTX-induced modification might not persist as in

the Na current experiment because the TTX molecule might somehow displace the BTX molecule from its binding site in the channel. As a result, the Na channel would return to the unmodified condition, exhibiting Na inactivation and gating charge immobilization. If this were the case, the Na current would exhibit inactivation after washing out TTX. Fig. 6 *C* shows that the removal of Na inactivation persisted after washing away TTX. This result has clearly ruled out the possibility that the presence of TTX had displaced BTX from its binding site and has unequivocally demonstrated that the gating charge immobilization observed after BTX treatment is not associated with unmodified Na channels.

Another possible type of interaction between BTX and TTX molecules which might contribute to the gating charge immobilization is the type of interaction involved between a local anesthetic and TTX molecule (Cahalan and Almers, 1979). The interaction between a local anesthetic and TTX is known to cause an immediate immobilization of gating charge. In addition, the decay of $I_{g,ON}$ and $I_{g,OFF}$ is greatly accelerated and the Q_{ON}–V relation at the positive potential is preferentially suppressed (see Figs. 8 and 10 of Cahalan and Almers, 1979). None of these features were seen with gating currents after BTX modification. Therefore, it is rather unlikely that the gating charge immobilization arises from the interaction between BTX and TTX. To test this hypothesis, one has to measure the gating current in the absence of TTX. However, such experiment turned out to be impossible when the Na channels had been modified by BTX, because there was too much ionic current flowing even in the absence of Na ions in the media, as shown in Fig. 6 *A*.

DISCUSSION

The main result presented in this paper is that whereas BTX, pronase, and chloramine-T could completely remove the fast Na channel inactivation, they affected differently the gating charge immobilization in squid giant axon. Both pronase and chloramine-T treatment abolished the gating charge immobilization concomitantly with the removal of the fast Na inactivation. In contrast to pronase and chloramine-T, BTX did not suppress the gating charge immobilization. We have demonstrated here that BTX uncouples the gating charge immobilization from the fast Na inactivation in squid axon.

Comparison with Previous Work

Our results with BTX on squid axon differ from the results previously obtained on the node of Ranvier. By integrating the $I_{g,OFF}$ at -120 mV, Dubois et al. (1983) have shown that after BTX treatment the Q_{OFF} (measured at -120 mV) after depolarizing pulses of 0 to $+20$ mV for various durations was 35% greater than Q_{ON} (measured at 0–20 mV). They concluded that BTX treatment has eliminated the gating charge immobilization. In contrast, by measuring the Q_{ON} associated with the test pulse in a double-pulse protocol where both conditioning and test pulses depolarized the membrane to the same potential $+20$ mV, we show that Q_{ON} decreased rapidly after depolarization, reaching ~40% of its initial value. Two points might help to explain the different outcomes of these two sets of experiments. First, the immobilized charge has been shown in squid axons to be "remobilized" by hyperpolarizing the membrane to very negative potential (Armstrong and Bezanilla, 1977). If such remobilization could also occur in the node of Ranvier, then the measurements of $I_{g,OFF}$ at -120 mV would not reveal the degree of charge immobilization that had occurred during the depolarizing potential. Second, as pointed out by Nonner (1980), there is a large variability in the $I_{g,OFF}$ measurements as a result of nonlinear leakage current, which makes the quantitative analysis of the total $I_{g,OFF}$ less reliable, especially after long depolarizing pulses. This could explain the increase in Q_{OFF} with the depolarizing pulse duration observed after BTX treatment for depolarizing pulses to 0 mV by Dubois et al. (see Fig. 2 *B* and Table 1 in Dubois et al., 1983). Of course, the different results obtained in the squid axon and in the Ranvier node could also be due to the species difference since other properties of the BTX-modified channels are found to be different in these two preparations, including the voltage-dependence of activation and the ionic selectivity.

Recently, choramine-T has been widely used as an agent to remove the Na inactivation in various preparations. The removal of Na inactivation with chloramine-T is in general more complete in squid axon (Wang et al., 1985; Huang et al., 1987) than in the Ranvier node (Wang, 1984*a*, *b*; Schmidtmayer, 1985; Rack et al., 1986; Meeder and Ulbricht, 1987; Neumcke et al., 1987). When removal of inactivation is complete, the gating charge immobilization essentially disappears in both preparations (Figs. 3 and 4; Drews, 1987). As pointed out by the author, the incomplete removal of the gating charge immobilization observed in some experiments in the Ranvier node could be explained by the presence of unmodified Na channels.

In squid axons, the effect of the removal of fast Na inactivation on the gating charge immobilization had been previously investigated only for pronase (Armstrong and Bezanilla, 1977). The fast Na inactivation and gating charge immobilization, which show similar monoexponential onset time course in untreated axon, are both suppressed by pronase treatment. In addition, once suppressed by pronase treatment, the gating charge immobilization can be restored by several agents which mimic inactivation, including pancuronium (Yeh and Narahashi, 1977; Yeh and Armstrong, 1978) and thiazine dyes (Armstrong and Croop, 1982). These observations have lent strong support to the notion that gating charge immobilization is closely linked to the fast Na inactivation.

Interpretation of our Results

The first model which gives an explicit kinetic and mechanical interpretation of both the fast Na inactivation and gating charge immobilization is commonly referred to as the ball-and-chain model for the fast Na inactivation (Armstrong and Bezanilla, 1977). In this model, the charge immobilization is considered to be strictly associated with the fast Na inactivation as a result of an electrostatic interaction between the activation gating particles (Q_x and Q_y) and inactivation particle (h-particle). During a step depolarization, the fast movement of the Q_x particle and the slow movement of Q_y particle through the membrane electrical field generate the fast and the slow component of $I_{g,ON}$, respectively, whereas, there is little or no charge movement associated with the movement of the h-particle. When the Q_y particle, which is though to have a charge opposite to that of the h-particle, moves towards the inner mouth of the channel, the channel mouth becomes attractive for the h-particle. This attraction between the channel and the h-particle can be depicted in terms of the binding of the h-gate to a specific receptor, the h-gate receptor. Kinetically, this postulated interaction between the Q_y particle and the h-particle decreases some of the backward rate constants governing the transitions by which the activation particles come out of the inactivated states upon repolarization. As a result, the gating charges return slowly to their original positions, resulting in a two-thirds to one-half loss of the charge movement, i.e., the gating charge immobilization (Armstrong and Bezanilla, 1977; Nonner, 1980; Stimers et al., 1985). In this view, inactivation and immobilization are closely linked and both processes are rate-limited by the binding of the h-particle to its receptor.

The removal of the fast Na inactivation and gating charge immobilization by pronase is interpreted as a consequence of the enzymatic removal of the h-particle (Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979; Stimers et al., 1985). The receptor for the h-gate is probably not modified since pronase has no effect on the activation or deactivation processes (Armstrong et al., 1973; Rojas and Rudy, 1976; Stimers et al., 1985), and since the charge immobilization can still be induced by the binding to the h-gate receptor itself, or to a closely related site, of molecules that simulate Na current inactivation (Yeh and Armstrong, 1978; Armstrong and Croop, 1982).

Like pronase, chloramine-T has no effect on the activation or deactivation process (Wang et al., 1985; Huang et al., 1987). Here, we have demonstrated that chloramine-T treatment removed both the fast inactivation and the gating charge immobilization. However, chloramine-T, being a mild oxidative agent, is thought to remove the fast Na inactivation differently from pronase (Wang et al., 1985; Rack et al., 1986). It remains to be tested whether the receptor for the h-gate is modified by chloramine-T treatment, e.g., whether the gating charge immobilization could be pharmacologically restored.

The gating charge immobilization of the BTX-modified channels exhibited several similarities with those seen in the control. First, the amount of charge that could be immobilized was similar (Figs. 2 *B* vs. 2 *D*; Fig. 4 *A*). Second, the shift of the Q_{ON}–V curve induced by the conditioning pulse to +20 mV (which causes Na current inactivation in the control axon) was identical in the control and in the BTX-modified channels (Fig. 5). Third, the voltage-dependence of the charge immobilization on prepulse amplitude was only slightly shifted in the BTX-modified channels (Fig. 4 *A*). However, the onset of immobilization in the BTX-modified channels was three times faster than in the control. Thus, the main effect of BTX is to accelerate the gating charge immobilization.

To interpret this BTX-induced charge immobilization in terms of the ball-and-chain model for the fast Na inactivation, we propose that BTX acts as an analogue of the endogenous ball, as far as the immobilization of the gating charge is concerned. We assume that in the BTX-treated channels the h-gate receptor is permanently occupied by BTX molecule, and that the presence of BTX on the h-gate receptor prevents the binding of the h-gate. The notion that BTX acts primarily on the receptor for the h-gate is supported by our previous results on the pharmacological properties of the BTX-modified channels. We have shown that the closing of the h-gate antagonizes the BTX modification of the Na channel, and reciprocally (Tanguy et al., 1984). However, unlike the inactivated channel, the BTX-bound channel is conducting since the BTX molecule does not occlude the channel. According to this model, it can be anticipated that the gating charge will be immediately immobilized after the Q_y particle moves to the inner mouth of the BTX-modified channel. Thus, the gating charge immobilization should follow the time-course of the transition association with the movement of Q_y particle. This notion is supported by the observation that the onset of gating charge immobilization (Fig. 2 *D*) has a time constant similar to that of the slow component of $I_{g,ON}$(300–500 μs) (Fig. 6 *B*), which is thought to reflect the movement of the Q_y particle.

Modeling of the Gating Charge Immobilization in BTX-Modified Channels

To simulate the relation between the fast Na inactivation and the gating charge immobilization for the control and for the BTX-modified channels, we use a simplified kinetic scheme of the original Armstrong and Bezanilla's model. In this scheme represented below, the movement of Q_y particle (associated with the transition $x_1 \leftrightarrow x_1 y$ of the original model) is represented by the transition $O_1 \leftrightarrow O_2$,

and the binding of the h-particle to its receptor by the transition $O_2 \leftrightarrow I$.

$$\begin{array}{ccccccc} & & CI_n & = & I & & \\ & & \| & & \| & & \text{slow} \\ C_{n-2} = & C_{n-1} = & C_n & = & O_1 & = & O_2 \\ & & & & \text{fast} & & \end{array}$$

Scheme II

where C_{n-2}, C_{n-1}, and C_n are various closed states of the channel, O_1 and O_2 are two conducting states, I represents the open inactivated state, and CI_n the closed inactivated state of the channel. In the control, the fast inactivation, which represents the transition $O_1 \leftrightarrow O_2 \leftrightarrow I$, is rate-limited by the binding of the h-particle to its receptor ($O_2 \leftrightarrow I$ transition). The gating charge immobilization, which has a similar time course to the fast inactivation one, occurs mainly because of the trapping of the Q_y particle by the h-gate bound to the h-gate receptor in the inactivated state. The onset of the charge immobilization is thus also rate-limited by the transition $O_2 \leftrightarrow I$. Since the $O_1 \leftrightarrow O_2$ transition is much faster than the $O_1 \leftrightarrow I$ transition, we simplify the kinetic scheme by lumping the two open states O_1 and O_2 into a single open state O. Thus the kinetic scheme for the normal channels becomes the following:

$$\begin{array}{ccccccc} & & CI_n & = & I & & \\ & & \| & & \| & & \text{slow} \\ C_{n-2} = & C_{n-1} = & C_n & = & O & & \end{array}$$

Scheme III: Normal Channels

According to this simple scheme, similar to the kinetic scheme of Armstrong and Gilly (1979), the onset of the charge immobilization, which reflects the transition $O \leftrightarrow I$, has the same time course as the inactivation process.

In the BTX-modified channel, since BTX is presumably permanently bound to the h-gate receptor, the Q_y particle is immediately trapped either by the charge BTX molecule itself or by the BTX-bound receptor. The transition $O_1 \leftrightarrow O_2$, which reflects the movement of the Q_y particle, becomes thus the rate-limiting step in the $O_1 \leftrightarrow I$ transition. The transition $O_2 \leftrightarrow I$ (Scheme II) does not involve anymore the binding of the h-particle and thus becomes much faster than the control $O_2 \leftrightarrow I$ transition. In addition, since BTX binding prevents the binding of h-gate to its receptor without occluding the channel, the I state becomes a conducting state O'. The kinetic scheme can be simplified by lumping the two open states O_2 and O' into a single open state O*. The simplest new kinetic scheme for the BTX-modified channels is thus

$$\begin{array}{ccccccc} & & CI_n & = & O^* & & \\ & & \| & & \| & & \text{fast} \\ C_{n-2} = & C_{n-1} = & C_n & = & O & & \end{array}$$

Scheme IV: BTX-modified channels

In this scheme, the C_{n-2} to O transitions must not be appreciably affected, since the $I_{g,ON}$ has a similar kinetics as in the control. The onset of the gating charge immobilization, which represents the transition $O \leftrightarrow O^*$, becomes rate-limited by the movement of the Q_y particle.

Upon repolarization, the return of the Q_y particle to its original position is slowed down by the electrostatic attraction between the Q_y particle and the BTX positively charged molecule, or the BTX-bound h-gate receptor. This slow return of the Q_y particle results in the immobilization of the gating charge. This also accounts for the slow tail currents experimentally observed upon repolarization in BTX-modified channels. In addition, after BTX-modifica-

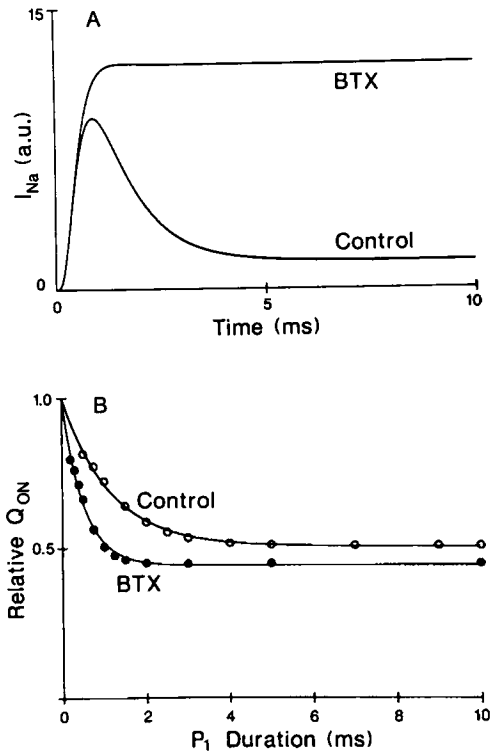

FIGURE 7 Na current inactivation and gating charge immobilization as predicted from the kinetic model for normal and BTX-modified channels. A modified scheme of Armstrong and Gilly (see Methods) was used to calculate the Na currents at +20 mV (A), and the onset time course of the gating charge immobilization (B) using the same double-pulse protocol method as in Fig. 2. The Na currents shown in A, elicited during a 10-ms pulse to +20 mV, decayed in control with a single exponential time course with a time constant of 1.04 ms, but failed to decay in BTX, as expected. Note that the rising phase of the BTX-modified current superimposes on the control one, as observed experimentally. To fit the charge immobilization time course, the $I_{g,ON}$ associated with the test pulse was generated for various durations of the conditioning pulse using the same double-pulse protocol as the one used in the experiments. The total Q_{ON} obtained by integrating $I_{g,ON}$ for 4 ms was normalized to its maximum value, and plotted as a function of the conditioning pulse duration in B. The onset time course of immobilization for control and BTX-modified channels was well fitted by a single exponential (continuous line) with a time constant of 1.01 and 0.44 ms, respectively. The nonimmobilizable component of the gating charges was 50% of its initial value in control and 43% in BTX-modified channels, values close to the experimental ones (see Fig. 2).

tion, the Na current tail decayed with a sum of two exponential function (Tanguy et al., 1985), as expected from the above scheme. (In our simulation, the fast Na tail is associated with the $O^* \leftrightarrow CI_n$ transition and the slow one associated with the closure of Na channel through the $O \leftrightarrow C_n$ pathway).

We have been able to simulate the gating charge immobilization and the removal of the fast Na inactivation using the kinetic scheme of Armstrong and Gilly (1979) with simple modifications. To obtain reasonably good fits of the Na currents kinetics and of the onset time course of the gating charge immobilization at +20 mV before and after BTX modification, several rate constants were empirically adjusted (the values are given in Table I). Fig. 7 *A* shows that the control Na current rises rapidly and then decayed monoexponentially with a time constant of 1.04 ms and fails to inactivate after BTX modification. Fig. 7 *B* shows that the onset time course of charge immobilization follows a single exponential time course with a time constant of 1.01 ms in control and 0.44 ms after BTX modification, and the nonimmobilizable component of the gating charges reaches a steady-state value of 50% of its initial value in control and 43% after BTX treatment. Thus, as experimentally observed, the gating charge immobilization in control has an onset time course similar to that of the inactivation of the Na current, while, after BTX treatment, the gating charge immobilization occurs with a fast time course close to that of the activation process. A more detailed kinetic scheme which can satisfactorily account for most of the kinetic and steady-state properties of both the Na currents and the gating currents of the BTX-modified channels will be presented elsewhere.

In conclusion, we have shown in the present paper that in squid axon (*a*) the gating charge immobilization and the fast Na inactivation are concomitantly suppressed by chloramine-T, (*b*) the fast Na inactivation and the gating charge immobilization can be pharmacologically uncoupled by BTX binding and (*c*) the charge immobilization is rate-limited by the inactivation process in channels with intact inactivation and by the activation process in BTX-modified channels.

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