QX-314 restores gating charge immobilization abolished by chloramine-T treatment in squid giant axons

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ABSTRACT The gating status of the QX-314 bound Na channels before and after suppressing the fast inactivation by chloramine-T (CT) was investigated by studying the gating charge immobilization using the OFF gating current ($I_{g,OFF}$). CT treatment, which abolishes the charge immobilization induced by a prolonged depolarization, altered the kinetics of $I_{g,OFF}$: the fast phase became insensitive to the pulse duration and the slow phase became three times faster than the control one. However, internally applied QX-314 (in the presence of external TTX) caused an immediate charge immobilization similar to that observed in the absence of CT treatment. The $I_{g,OFF}$ exhibited kinetics similar to the inactivated channels, decaying with a very fast time course. We conclude that the charge immobilization is restored by QX-314 in the chloramine-T-treated axon and that the gating state of the QX-314-bound channel is similar to the inactivated one. The role of the gating charge immobilization in the use-dependent block mechanism is discussed.

INTRODUCTION

Three lines of evidence support the notion that the inactivation gate plays an important role in the Na channel blocking action by local anesthetics (Hille, 1977). First, the inactivation is enhanced by many local anesthetics (Hille, 1977). Second, use-dependent blocked Na channels resemble inactivated Na channels in that the gating charge immobilization occurs in both cases (Cahalan and Almers, 1979). Third, the removal of the fast Na inactivation by pronase or *N*-bromoacetamide completely or partially reduces the potency of the quaternary lidocaine derivative QX-314 to block the Na currents, primarily resulting from a loss of use-dependent block of Na channels in response to a train of depolarizing pulses (Cahalan, 1978; Yeh, 1978). In addition, after pronase treatment, QX-314 becomes unable to immobilize the gating charge movement (Yeh, 1982). However, recent experiments with chloramine-T (CT) have demonstrated that despite the complete removal of the fast inactivation in squid axons (Wang et al., 1985; Huang et al., 1987), or its partial removal in myelinated nerve fibers (Wang, 1984), the use-dependent block of Na currents by QX-314 is extant (Shepley et al., 1983; Strichartz and Wang, 1986; Wang et al., 1987; Yeh and Tanguy, 1986). These results, as pointed out by their authors, put into question the predominant role of the fast inactivation in local anesthetics blocking action as originally proposed in the modulated receptor hypothesis (Hille, 1977). Alternatively, CT could remove the fast inactivation by a mechanism different from pronase such that, after CT treatment, the QX-314–bound channels could still undergo the transition to the inactivated state, whereas after pronase treatment, they could not undergo such a transition.

To test this hypothesis further, we used the gating current measurements to determine whether the QX-314–bound channel was still similar to the inactivated channel after the removal of the fast inactivation by CT. We found that after the suppression of both the fast inactivation and the gating charge immobilization by CT treatment, QX-314 could restore the gating charge immobilization to the same extent as in an axon with intact inactivation. We conclude that after CT treatment the gating state of the QX-314–bound channels is similar to the inactivated one and that this drug-bound inactivated state plays an important role in the QX-314 use-dependent block mechanism.

METHODS

Experiments were performed on giant axons isolated from squids, *Loligo pealei*, obtained at the Marine Biological Laboratory (Woods Hole, MA). Axons were internally perfused with 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 were positioned on both sides of the axon and air gaps were created on both ends of the axon to improve the space clamp (Oxford, 1981). Feedback circuit for the series resistance compensation was used to compensate for errors arising from series resistance. The Na channel current was measured by the outward-going Cs current through the Na channels in the axon externally perfused with a Na-free artificial sea water containing (in millimolar): tetramethylammonium, 450; Ca^{2+}, 50;

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Cl^-, 540; Hepes, 10 (pH 7.3); and internally with a Na-free solution contained (in millimolar): Cs^+, 300; glutamate$^-$, 240; F^-, 50; sucrose, 400; MOPS buffer, 10 (pH 7.3). The removal of the fast inactivation of the Na channel with internally applied CT was monitored by the disappearance of the decaying phase of Cs current through the Na channels. A freshly prepared solution containing 5 mM CT was internally applied to the axon for ~10 min (Huang et al., 1987), and was subsequently washed out thoroughly. For the gating current measurements, 2 μM tetrodotoxin (TTX) was added to the externally solution to eliminate ionic currents through the Na channels. All experiments were performed at temperature 8–9°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 intervals by a 14-bit analog-to-digital converter. To obtain the voltage dependence of the gating charge movement (Q_{ON}-E_m curve), both the P − P/4 and P + P/4 methods were used the P − P/4 protocol for the depolarizing pulse (Armstrong and Bezanilla, 1974) and the P + P/4 protocol for the hyperpolarizing pulse so that the P/4 pulse was always stepping in the direction more negative than the shifting potential of −150 mV. The ON gating current ($I_{g,ON}$) associated with each pulse was integrated for 2.5 ms to obtain the total charge movement (Q_{ON}). The gating charge immobilization was studied by measuring the OFF gating current ($I_{g,OFF}$) upon returning the membrane to −80 mV after a depolarizing pulse to +20 mV for various durations (Armstrong and Bezanilla, 1977). The kinetics of $I_{g,OFF}$ were analyzed by fitting to the data points using a least-square algorithm for simultaneous multiexponential fit. The total charge movement (Q^t_{OFF}) associated with $I_{g,OFF}$ was directly integrated from $I_{g,OFF}$ and the fast component (Q^f_{OFF}) of $I_{g,OFF}$ was calculated from the curve fitting program. The onset of the gating charge immobilization was monitored with the decrease of the Q^f_{OFF} in the inactivation intact axon (Nonner, 1980) and with the decrease of the Q^t_{OFF} in the CT-treated axon.

RESULTS

QX-314 restored the gating charge immobilization abolished by CT treatment

The alteration by internal application of 1 mM QX-314 of the gating charge immobilization determined from the kinetics of the OFF gating currents ($I_{g,OFF}$) was compared before and after CT treatment (after washing away CT). The $I_{g,OFF}$ after a depolarizing pulse to +20 mV of various durations were recorded upon repolarizing the membrane to the holding potential of −80 mV (Fig. 1).

In an axon with intact inactivation (Fig. 1, *A* and *B*), both the kinetics and the amount of the charge movement associated with the $I_{g,OFF}$ (Q_{OFF}) varied with the pulse duration (Fig. 1 *A*, a). After a short depolarizing pulse (0.5–1 ms), the $I_{g,OFF}$ decayed mainly with a single exponential time course with an averaged time constant of ~150 μs (Table 1). As the pulse duration was lengthened, the $I_{g,OFF}$ time course became biexponential and the time constant of the fast component (τ_f) of $I_{g,OFF}$ decreased with increasing the pulse duration. As shown in Table 1, τ_f decreased to ~100 μs for the pulses >4 ms. In addition, the slow component of $I_{g,OFF}$ which was negligibly small after a short depolarizing pulse (Fig. 1 *B*, a) became larger as the pulse duration was lengthened (Fig. 1 *B*, b) so that 60% of the charge was recovered from the slow component. The time constant for the slow component ranged from 1 to 3 ms, with an average of ~1.5 ms (Table 1). During the internal application of QX-314 (Fig. 1 *A*, b), the $I_{g,OFF}$ for the short depolarizing pulses (≤1 ms) was speeded up to the extent that its τ_f value was similar to that measured for long depolarizations in the control (Table 1), whereas the $I_{g,OFF}$ for the long depolarizing pulses was almost superimposable on the control trace (Fig. 1 *B*, b). The slow component was negligible after 0.5 ms pulse (Fig. 1 *B*, a), and became very prominent after 10 ms pulse (Fig. 1 *B*, b) as seen in the control.

These alterations of the $I_{g,OFF}$ by QX-314 observed after a single depolarizing pulse were identical to those observed after a train of depolarizing pulses. As observed by Cahalan and Almers (1979), the presence of external TTX stabilizes the QX-314 molecule at its blocking site so that the block is no longer use-dependent, in contrast to the use-dependent block of the Na current always observed in absence of TTX.

In the CT-treated axon, QX-314 altered both the kinetics and amount of charge associated with the $I_{g,OFF}$ (Fig. 1, *C* and *D*). After the removal of the gating charge immobilization by CT, $I_{g,OFF}$ did not decrease in amplitude (Fig. 1 *C*, a). However, both components of the $I_{g,OFF}$ were affected by the CT treatment (Fig. 1 *D*, a and b): the fast components of $I_{g,OFF}$ became insensitive to the duration of the depolarizing pulse with the τ_f values slightly smaller than the control ones, whereas the averaged time constant of the slow component, which was still increasing with the pulse duration, was almost three times smaller than that seen after long depolarizing pulse before CT

TABLE 1 Effects of QX-314 on the time constants of the OFF gating currents in axons with intact inactivation and after CT treatment

	Pulse duration *ms*	τ_f μs	τ_s μs	No.
Inactivation intact axons				
Control	≤1	148 ± 20	Negligible	10
	≥4	102 ± 12	1470 ± 420	10
QX-314	≤1	116 ± 13	Negligible	3
	≥4	98 ± 9	1650 ± 960	3
CT-treated axons				
Control	≤1	114 ± 22	Negligible	8
	≥4	116 ± 12	510 ± 89	8
QX-314	≤1	74 ± 29	Negligible	5
	≥4	72 ± 10	481 ± 132	5

QX-314 was internally applied to the axon at 1 mM. The OFF gating currents were fitted to a sum of two exponential functions with a fast time constant (τ_f) and a slow time constant (τ_s).

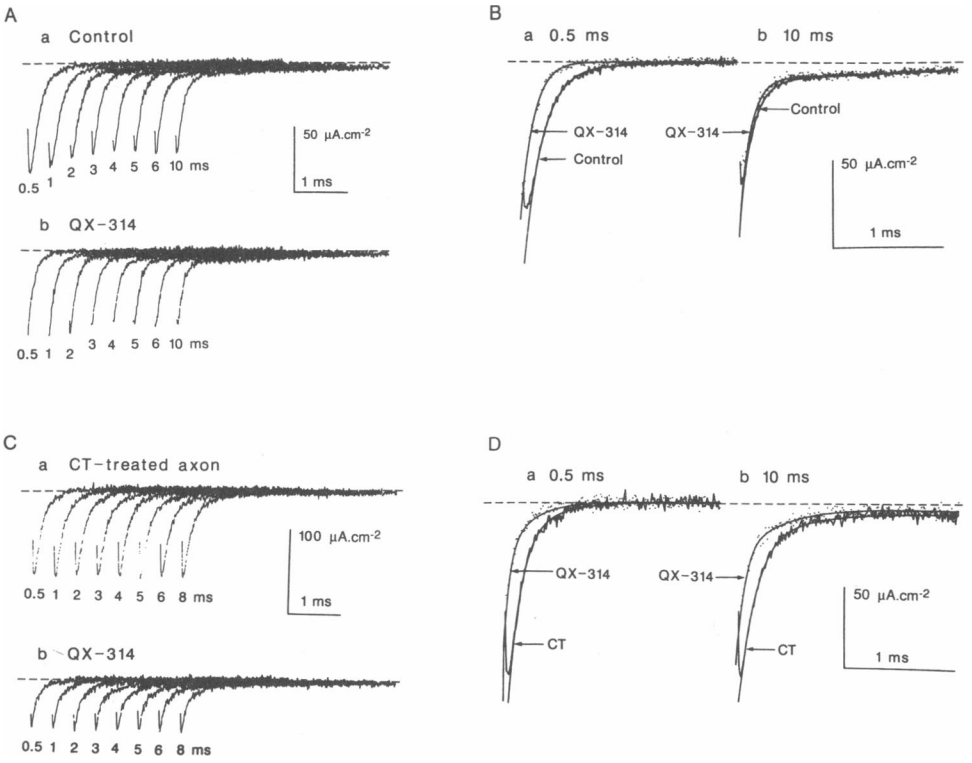

FIGURE 1 QX-314–induced gating charge immobilization before and after CT treatment. The OFF gating current ($I_{g,OFF}$) was obtained by returning the membrane to −80 mV after the indicated duration of depolarizing pulse to +20 mV. (*A*) In the axon with intact inactivation, $I_{g,OFF}$ gradually diminished in size and changed in kinetics as the pulse duration was lengthened in control (a). In the presence of 1 mM QX-314, such changes occurred even for the depolarizing pulse as short as 0.5 ms (b). (*B*) Kinetic analysis of the $I_{g,OFF}$ showed the first 2 ms curve fitting by a sum of two exponentials. In the control, after 0.5 ms pulse (*B*, a) 27 nC/cm^2 of the charge moved during $I_{g,OFF}$ with a fast time constant (τ_f) of 171 μs and 4.3 nC/cm^2 with a slow time constant (τ_s) of 731 μs. In the presence of 1 mM QX-314, 13 nC/cm^2 decayed with τ_f of 110 μs and 4.6 nC/cm^2 with a τ_s of 1.47 ms. After a 10-ms pulse (*B*, b), the control $I_{g,OFF}$ had 13 nC/cm^2 charge moved with a τ_f of 114 μs and 20 nC/cm^2 moved with a τ_s of 3 ms. In the presence of 1 mM QX-314, 11 nC/cm^2 of charge moved with τ_f of 94 μs and 6.5 nC/cm^2 with a τ_s of 4.4 ms. (*C*) After CT treatment, $I_{g,OFF}$ remained unchanged in amplitude as the pulse duration was lengthened (*C*, a). Internally applied 1 mM QX-314 to the CT-treated axon caused an immediate reduction in $I_{g,OFF}$ and changes in its decaying kinetics (*C*, b). (*D*) Curve fitting to the CT trace after 0.5 ms depolarizing pulse (*D*, a) showed that 21 nC/cm^2 charge moved with a τ_f of 115 μs and 10 nC/cm^2 moved with a τ_s of 437 μs. In 1 mM QX-314, 4 nC/cm^2 charge moved with a τ_f of 50 μs and 9 nC/cm^2 with a τ_s of 250 μs. After a 10-ms depolarizing pulse (*D*, b). 15 nC/cm^2 charge moved with a τ_f of 119 μs and 15 nC/cm^2 moved with a τ_s of 562 μs. In the presence of 1 mM QX-314, 4 nC/cm^2 charge moved with a τ_f of 55 μs and 13 nC/cm^2 with τ_s of 441 μs.

treatment (Table 1). During internal application of 1 mM QX-314, $I_{g,OFF}$ was reduced in amplitude and the reduction was almost identical regardless of the pulse duration (Fig. 1 *C*, b). The curve fitting to the data traces after 0.5 and 10 ms depolarizing pulses is illustrated in Fig. 1 *D*. The most prominent feature of the $I_{g,OFF}$ in the presence of QX-314 was the very fast time course of its decaying phase, mainly resulting from the reduction of the time constant of the fast component (Table 1).

Comparison between the QX-314- and inactivation-induced gating charge immobilization

Because the changes in the kinetics and the amplitude of $I_{g,OFF}$ reflect the gating charge immobilization, the τ_f values and the OFF charge movement (Q_{OFF}) were plotted as a function of the duration of depolarizing pulse to monitor the time course of development of the immobilization process (Fig. 2). In the control (Fig. 2 *A*), τ_f decreased as the pulse duration was lengthened, reaching a constant value 50% smaller than that measured for a 0.5-ms pulse. In the presence of 1 mM QX-314 the τ_f of $I_{g,OFF}$ after a 0.5-ms depolarizing pulse resembled the value seen in the control for the long pulse duration (see also Table 1). After CT treatment, $I_{g,OFF}$ was no longer sensitive to the duration of the depolarizing pulse (Fig. 2 *B*). Internal application of 1 mM QX-314 reduced τ_f values which were still insensitive to the pulse duration, and the degree of reduction in τ_f was similar to that seen in the axon with Na inactivation for 0.5 ms pulse duration (Fig. 2 *A*).

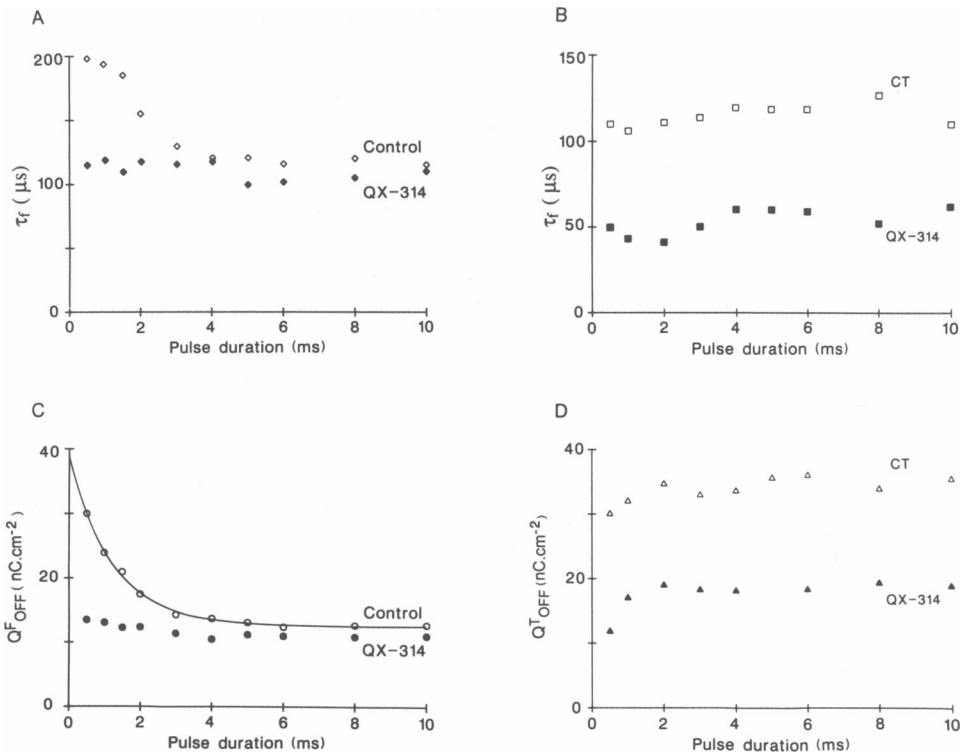

FIGURE 2 QX-314 caused a similar charge immobilization before (*A* and *C*) and after the removal of the fast Na inactivation by CT (*B* and *D*). (*A*) The fast time contant τ_f diminished progressively with the pulse duration in control but was reduced to a rather constant value for a pulse as short as 0.5 ms in the presence of 1 mM QX-314. (*B*) After CT treatment, the τ_f of $I_{g,OFF}$ remained constant and reduced to ~40% of the control by 1 mM QX-314. (*C*) Prolongation of depolarizing pulse to +20 mV diminished the Q^f_{OFF} with a time constant of 1 ms, and reduced its value to 30% of the original level. QX-314 caused an immediate reduction of Q^f_{OFF}. (*D*) The Q^t_{OFF} remained unchanged after CT treatment but was reduced to 60% of the control level by 1 mM QX-314.

The decrease in the fast component of $I_{g,OFF}$ (Q^f_{OFF}) was used to monitor the extent of the gating charge immobilization in axons with intact inactivation (Nonner, 1980) (Fig. 2 *C*). The Q^f_{OFF} decreased with a single exponential function of the pulse duration with a time constant of 1 ms, a value similar to that for the Na current inactivation measured at the same potential in the same axon before TTX application. The steady-state value of Q^f_{OFF}, representing the inactivation-resistant component of the gating charge movement (Armstrong and Bezanilla, 1977), corresponded to 30% of its initial value. In the presence of QX-314, Q^f_{OFF} did not decrease anymore as a function of pulse duration, and was instead reduced to a value close to the control steady-state one (Fig. 2 *C*). After CT treatment, the $I_{g,OFF}$ decayed back to the baseline level within 4 ms and the total charge movement (Q^t_{OFF}) could be directly integrated from $I_{g,OFF}$ to monitor the degree of charge immobilization. The Q^t_{OFF} did not decrease as a function of pulse duration (Fig. 2 *D*), confirming that CT treatment has indeed removed the gating charge immobilization. Internal QX-314 application (1 mM) caused a constant reduction of Q^t_{OFF} which was independent of the pulse duration (Fig. 2 *D*), as observed in axons with intact inactivation (Fig. 2 *C*).

We further investigated the similarity between QX-314–induced and inactivation-induced charge immobilization by studying the voltage dependence of the total ON gating charge movement (Q_{ON}) obtained by integrating the ON gating currents (Fig. 3). First, we compared the effects of 1 mM QX-314 on the voltage dependence of the gating charge movement before and after CT treatment (Fig. 3, *A* and *B*). In both the control (Fig. 3 *A*) and CT-treated axon (Fig. 3 *B*), QX-314 decreased the total Q_{ON} only at membrane potentials more positive than −60 mV. At +20 mV, for example, QX-314 caused an average reduction (mean ± SD) in the Q_{ON} of 45 ± 10% (n = 3) in the control and 43 ± 10% (n = 5) in the CT-treated axon. This effect was reversible upon washing away QX-314. Thus, the removal of the fast inactivation by CT did not seem to affect the blocking action of QX-314 on the gating current. Second, we compared the voltage-dependence of Q_{ON} obtained in an axon with intact inactivation after applying a conditioning potential to induce the Na current inactivation and the gating

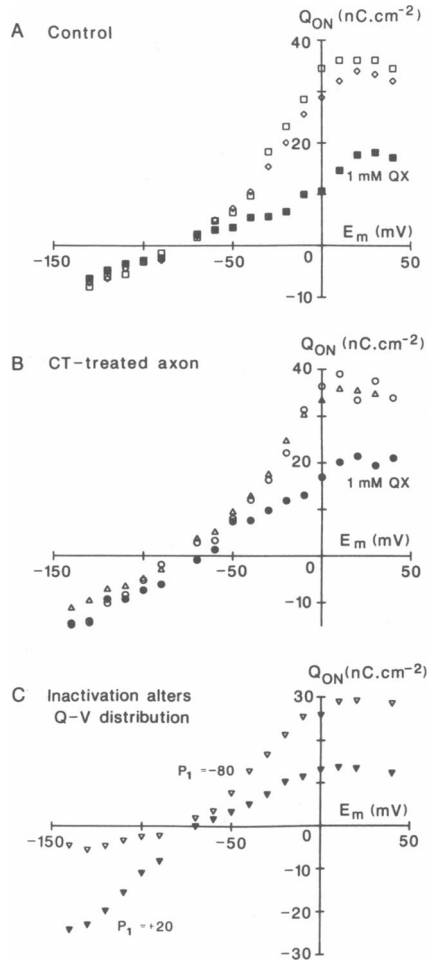

FIGURE 3 Comparison of the effects of QX-314 and of the fast inactivation on the Q_{ON}-E_m distribution. QX-314 at 1 mM reversibly suppressed the Q_{ON} in control and CT-treated axons. (*A*) In an axon with intact inactivation (control), the Q_{ON}-E_m recorded before (□), during (■), and after (◊) washing away QX-314 shows that the Q_{ON} associated with depolarizing pulses >-60 mV was suppressed by QX-314 to ~50% of the control. (*B*) A similar effect of QX-314 was seen in the CT-treated axon: before (○), during (●), and after (△) washing away 1 mM QX-314. (*C*) In an axon with intact inactivation, in the absence of QX-314, the Q_{ON}-E_m curve obtained without any conditioning pulse ($P_1 = -80$ mV) (▽) was compared to that obtained after a 10-ms conditioning depolarizing pulse ($P_1 = +20$ mV) (▼).

charge immobilization (Armstrong and Bezanilla, 1977; Bezanilla et al., 1982) (Fig. 3 *C*), with those obtained during QX-314 application in an axon with intact inactivation on one hand (Fig. 3 *A*), and in a CT-treated axon on the other hand (Fig. 3 *B*). Fig. 3 *C* shows that a 10-ms depolarizing pulse to +20 mV decreased Q_{ON} by >50% at potentials more positive than −70 mV, whereas it increased Q_{ON} at potentials more negative than −80 mV. In contrast, the 45% decrease of Q_{ON} induced by QX-314 at potentials more positive than −70 mV was not accompanied by an increase of Q_{ON} at potentials more negative than −80 mV, regardless of whether the fast inactivation had been removed by CT treatment (Fig. 3, *A* and *B*).

DISCUSSION

By examining the effect of QX-314 on the Na gating current, we found that (*a*) QX-314 induced the immobilization of the gating charges which had been removed by CT treatment, (*b*) the QX-314–induced gating charge immobilization was similar in the CT-treated axon and in the axon with intact inactivation, and (*c*) the QX-314–induced gating charge immobilization resembled the inactivation-induced gating charge immobilization.

Using a double pulse protocol to measure the ON gating current associated with the test pulse after various durations of a conditioning depolarizing pulse, Tanguy and Yeh (1988) have demonstrated that CT, which removes the Na current inactivation, abolishes the gating charge immobilization in squid axons. The removal of charge immobilization by CT was illustrated here as the alteration of the amplitude and kinetics of $I_{g,OFF}$. The $I_{g,OFF}$ amplitude became constant, and the fast time constant τ_f was unaltered when the duration of the depolarizing pulse was increased. A similar effect of the removal of the fast Na inactivation on $I_{g,OFF}$ was seen with pronase in squid axons (Armstrong and Bezanilla, 1977) and with CT in frog nodes of Ranvier (Drews, 1987).

The QX-314–induced gating charge immobilization (characterized in the presence of external TTX to suppress ionic currents) resembled the fast inactivation-induced charge immobilization in many respects. First, as illustrated in Fig. 2 *D*, the maximal amount of the charge that could be immobilized is almost identical in both cases, being ~60% of the total charge movement (Cahalan and Almers, 1979; Greef et al., 1982). Second, the kinetics of the ON gating current in the presence of QX-314 resembles the kinetics, in the absence of drug, of the inactivated channels (Fig. 10 in Cahalan and Almers, 1979). Here we show that the fast component of $I_{g,OFF}$ in the presence of QX-314 had similar kinetics as observed in the control after the channels had been inactivated (Fig. 1 *B*, b). These results suggest that both QX-314 and Na inactivation affect the same component of the gating charge movement (Cahalan and Almers, 1979). In other words, the gating state of the QX-314–bound channels is similar to the inactivated state.

However, the Q_{ON}-E_m curve for the inactivated Na channels exhibited some differences with that obtained for the QX-314–bound channels. Although at potentials more positive than −60 mV the Q_{ON}-E_m curve was characterized by a decrease of Q_{ON} in both cases (see Fig. 3, *A*–*C*), at potentials more negative than −80 mV the

Q_{ON}-E_m curve obtained in the presence of QX-314 was not significantly affected (Fig. 3, *A* and *B*), whereas the Q_{ON}-E_m curve for the Na channel inactivated by the fast inactivation was characterized by an increase in the absolute value of Q_{ON} (see also Armstrong and Bezanilla, 1977; Bezanilla et al., 1982) (Fig. 3 *C*). Such an increase in the Q_{ON} at potentials more negative than −70 mV is explained by the fast recovery from Na channel inactivation when the membrane is hyperpolarized (Bezanilla and Armstrong, 1977; Yeh and Tanguy, 1985). As a result, the inactivation-immobilized charge can be remobilized at faster rate at hyperpolarizing potentials (Armstrong and Bezanilla, 1977; Bezanilla et al., 1982). Thus, hyperpolarizing the membrane can recover the inactivation-induced charge immobilization. In contrast, when the membrane is made more hyperpolarized, the QX-314 bound Na channels remain in the bound state longer because hyperpolarization slows the rate at which the QX-314 molecule could dissociate from the channel (Yeh and Tanguy, 1985). As a result, the QX-314–induced charge immobilization could not be recovered by hyperpolarizing the membrane.

In response to a train of depolarizing pulses, QX-314 caused a pronounced use-dependent block of Na current in CT-treated axons with an intensity almost matching that seen in the axon with Na inactivation (Wang et al., 1987; Yeh and Tanguy, 1986), whereas QX-314 does not cause a pronounced use-dependent block of Na current in pronase-treated axons (Cahalan, 1978; Yeh, 1978; Wang et al., 1987) nor cause the gating charge immobilization (Yeh, 1982). This difference in the effect on the gating charge immobilization may provide an explanation for the difference in the use-dependent block of Na currents between the pronase- and CT-treated axons. The fast component of $I_{g,OFF}$ is thought to reflect the closure of the channel (Armstrong and Croop, 1982) and the slow component is attributed to the inactivation of the channel (Armstrong and Bezanilla, 1977) resulting from an interaction between the inactivation gate and its receptor (Stimers et al., 1985). Inasmuch as the fast component of $I_{g,OFF}$ is not affected by QX-314, the activation gate of the drug-bound channel is probably able to close normally. These results indicate that when the activation gate closes, the QX-314 molecule is being trapped in the channel, and the trapped molecule escapes from the channel slowly, manifesting as the use-dependent block of Na currents. Because the slow component of the OFF gating current is extant in the presence of QX-314 in the axon with normal inactivation or in the CT-treated axon, QX-314 must have induced the channel into the inactivated state. The inactivated state would help to stabilize the drug-channel complex, thereby contributing to the extent of the use-dependent block. Thus, both the activation mechanism and the ability of the drug-bound channels to undergo a transition to an inactivated state are required for producing a full-blown use-dependent block of Na channels by local anesthetics.

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