SAXITOXIN AND TETRODOTOXIN.

ELECTROSTATIC EFFECTS ON SODIUM CHANNEL GATING CURRENT IN CRAYFISH AXONS

STEVEN T. HEGGENESS AND JOHN G. STARKUS
Department of Physiology, John A. Burns School of Medicine, and the Bekesy Laboratory of Neurobiology, Pacific Biomedical Research Center, University of Hawaii, Honolulu, Hawaii 96822

ABSTRACT The effects of extracellular saxitoxin (STX) and tetrodotoxin (TTX) on gating current (I_gON) were studied in voltage clamped crayfish giant axons. At a holding potential (V_H) of −90 mV, integrated gating charge (Q_{ON}) was found to be 56% suppressed when 200 nM STX was added to the external solution, and 75% suppressed following the addition of 200 nM TTX. These concentrations of toxin are sufficiently high to block >99% of sodium channels. A smaller suppression of I_gON was observed when 1 nM STX was used (K_D = 1–2 nM STX). The suppression of I_gON by external toxin was found to be hold potential dependent, with only minimal suppression observed at the most hyperpolarized hold potentials, −140 to −120 mV. The maximal effect of these toxins on I_gON was observed at hold potentials where the Q_{ON} vs. V_H plot was found to be steepest, −100 to −80 mV. The suppression of I_gON induced by TTX is partially relieved following the removal of fast inactivation by intracellular treatment with N-bromoacetamide (NBA). The effect of STX and TTX on I_gON is equivalent to a hyperpolarizing shift in the steady state inactivation curve, with 200 nM STX and 200 nM TTX inducing shifts of 4.9 ± 1.7 mV and 10.0 ± 2.1 mV, respectively. Our results are consistent with a model where the binding of toxin displaces a divalent cation from a negatively charged site near the external opening of the sodium channel, thereby producing a voltage offset sensed by the channel gating apparatus.

INTRODUCTION

Extracellular application of tetrodotoxin (TTX) blocks the voltage dependent conductance pathway for sodium ions (Narahashi et. al., 1964; Moore and Narahashi, 1967). Subsequent studies have demonstrated that saxitoxin (STX) also blocks sodium channels, and a similar mechanism of action has been proposed for both toxins (Kao and Nishiyama, 1965; Hillie, 1975). During the past twenty years, these cationic toxins have been used extensively to block ionic current through the sodium channels, facilitating the study of potassium currents and also the intramembranous gating charge movements associated with sodium channel opening (I_gON). Although TTX has been shown to slow the recovery of inactivated sodium channels in rabbit Purkinje fibers (Cohen et al., 1981), gating currents recorded from squid axon have been reported to be essentially unaffected by blocking concentrations of TTX (Armstrong and Bezanilla, 1974; Gilly and Armstrong, 1982) or STX (Keynes and Rojas, 1974). Based on these observations, it has been generally assumed that the binding of these cationic toxins does not affect sodium channel gating in axon preparations.

Address Correspondence to Dr. John G. Starkus, Bekesy Laboratory of Neurobiology, Pacific Biomedical Research Center, University of Hawaii, 1993 East West Road, Honolulu, Hawaii 96822.
USENET: (ihnp4!islenet!bigtuna!john)

On the other hand, several studies have demonstrated that high concentrations of external divalent cations decrease sodium channel block by STX and TTX (Henderson et al., 1973; Henderson et al., 1974; Hille et al., 1975a). Because divalent cations interact with these toxins at their binding site near the mouth of the sodium channel, it is reasonable to suspect that toxin binding may displace divalent cations from this site. This suggestion is partially supported by the recent observation of Gilly and Armstrong (1982) that pre-treatment with TTX antagonizes the effect of external Zn^{++} on sodium channel gating current. Since it is well established that the external divalent cation concentration affects sodium channel kinetics (e.g., Frankhaeuser and Hodgkin, 1957; Dodge, 1961; Hille et al., 1975*b*), and since both STX and TTX compete with these divalent cations, it is surprising that binding of these cationic toxins apparently fails to affect channel kinetics.

In the present study we have carefully compared gating currents in the presence and absence of external STX and TTX. In contrast to the lack of effect previously reported for squid axons, we note significant effects of these toxins on sodium channel gating currents and steady state inactivation in crayfish giant axons. A preliminary report of this work was presented in abstract form at the XXIX annual Biophysical Society meeting (Starkus and Heggeness, 1985).

METHODS

Preparation and Data Recording

Medial giant axons from the crayfish, *Procambarus clarkii,* with diameters between 200 and 300 μM, were internally perfused and voltage clamped using methods previously described (Shrager, 1974; Starkus and Shrager, 1978; Starkus et al., 1981). Series resistance was compensated at 10 ohm-cm^2 and corrections were made for an electrode junction potential of 8–10 mV. Temperature was controlled and maintained at 6 ± 0.1°C. Data traces were digitized with 12-bit resolution at a sample rate of 0.5 to 2 μS using a Nicolet digital oscilloscope (model 1090A; Nicolet Instrument Corp., Madison, WI) and signal averaged on a Nicolet 1170. Signal averaged records then were transferred to a microcomputer (model OSI C3-S1; Ohio Scientific Inc., Aurora, OH) for analysis and storage. Data were plotted for presentation using a digital plotter (model HI PLØT; Houston Instruments, Austin, TX). Due to the resolution of the plotter, traces were typically plotted using every fifth data point.

Pulse Programs

Linear capacity current and leakage currents were subtracted directly on the Nicolet 1170 signal averager by utilizing a −P/2 protocol, with hyperpolarizing pulses from a 10 ms conditioned base potential of −140 or −150 mV to a maximum of −220 mV. These base potentials are sufficiently negative to avoid any asymmetric charge movement in the hyperpolarizing pulses. Due to the presence of a slow linear capacity current, which outlasts the rise time of the voltage steps, the durations of the hyperpolarizing pulses were exactly matched to the test pulse durations. Fig. 1 demonstrates that in the voltage range −150 to −200 mV, where no gating charge movement is detected, these pulse programs result in complete and accurate subtraction of the linear capacity and leakage currents.

Gating currents were measured by signal averaging 16 consecutive depolarizing test pulses at a frequency of 0.5 Hz followed by 32 consecutive hyperpolarizing pulses, previously calculated to match the divided pulse protocol (−P/2). To insure that our observed results were not in any way influenced by frequency-dependent effects on the sodium channels, the interpulse interval was varied from 2–30 s and no differences were observed in either the kinetics of gating charge movement or in the effects of external toxin on steady state inactivation.

Solutions and Reagents

The composition of our external and internal solutions are summarized in Tables I and II. Following the convention of Armstrong and Bezanilla (1974), solution information is listed below as external solution//internal solution (i.e., 0 Na MVH//230 TMA). Reagents utilized in this study include STX, TTX, pronase from *Streptomyces griseus* type XIV (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA) and *N*-bromoacetamide (NBA) (Sigma Chemical Co., St. Louis, MO).

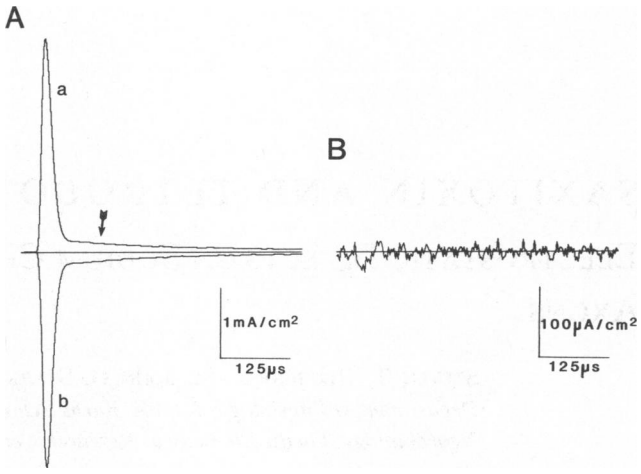

FIGURE 1 Direct summation of comparable hyperpolarizing and depolarizing pulses results in complete subtraction of I_{Cap} and linear leakage current in crayfish axons. In *A*, membrane currents associated with voltage steps from −200 to −150 mV (*a*) and −150 to −200 mV (*b*). A slow component of capacity current is identified in these records by the arrow. In *B*, summation of the positive and negative pulses demonstrates the accuracy of the method and linearity of the membrane preparation. Axon 022885, 0 Na MVH//230 TMA.

RESULTS

External STX Suppresses I_gON

A comparison of sodium channel gating current before and after application of STX demonstrates that I_gON is suppressed by external STX. This suppression of gating current is holding potential sensitive, and occurs without any visible change in the kinetics of I_gON (as will be discussed below). To prevent sodium current from contaminating I_gON in the absence of external STX, gating currents were collected in 230 Cs internal perfusate (see Table II) at the sodium channel reversal potential (E_{Rev}). In addition, perfusion with 230 Cs (K^+ free) solution results in a complete blockade of ionic current through potassium channels in crayfish axons, permitting these experiments to be conducted without the use of other potassium channel blocking agents.

The results shown in Fig. 2 reveal that with a holding potential of −90 mV, the degree of suppression of I_gON is

TABLE I
EXTERNAL SOLUTIONS

Name	Na^+	K^+	Mg^{++}	Ca^{++}	TMA^+	Cl^-	Hepes	pH
				mM				
NVH*	210	5.4	2.6	13.5	0	247.6	2	7.55
0 Na MVH‡	0	0	2.6	13.5	210	242.2	2	7.55
210 Na MVH‖	210	0	2.6	13.5	0	242.2	2	7.55

*(NVH) normal Van Harreveld solution (Van Harreveld, 1936).
‡(MVH) modified Van Harreveld solution.
‖Low external sodium solution achieved by appropriate mixing of 210 Na MVH with 0 Na MVH.

TABLE II
INTERNAL SOLUTIONS

Name	K^+	Cs^+	Na^+	TMA^+	F^-	glutamate$^-$	Hepes	pH
					mM			
250 K	250	0	0	0	60	190	1	7.35
230 Cs	0	230	0	0	60	170	1	7.35
230 Na*	0	0	230	0	60	170	1	7.35
230 TMA	0	0	0	230	60	170	1	7.35

All internal and external solutions checked for osmolarity in the range 430–440 mosM.
*Low internal sodium achieved by appropriate mixing of 230 Na and 230 TMA.

dependent on STX concentration. For this figure, gating currents were measured at E_{Rev}, +26 mV, while sodium currents (I_{Na}) were measured at a test potential of −10 mV. In this way, the fraction of sodium channels blocked at each dose of STX could be directly evaluated, and compared with the degree of suppression observed in I_gON. In Figure 2 *A*, the peak of I_gON in the absence of toxin was 162 $\mu A/cm^2$, with a total charge moved (Q_{ON}) of 20 nC/cm^2. At a low dose of 0.1 nM STX, I_{Na} at −10 mV was not noticeably reduced, and no suppression of I_gON was seen (not shown). When external STX concentration was raised to 1 nM (*B*), I_{Na} was 49% blocked, peak I_gON was reduced to 127 $\mu A/cm^2$, and Q_{ON}, was reduced to 15 nC/cm^2. Increasing the dose of STX to 10 nM (*C*) completely blocked I_{Na} and further reduced peak I_gON to 90 $\mu A/cm^2$ and Q_{ON} to 8 nC/cm^2. The STX record (*C*) can be scaled by 1.8× to superimpose the control record (*A*), confirming that ionic current contamination in these records is minimal. This suppression of gating current is reversible with washout of external toxin. After a 15-min washout of STX (*D*), 80% of I_{Na} at −10 mV was recovered, peak I_gON returned to 167 $\mu A/cm^2$, and Q_{ON} increased to 19 nC/cm^2. Subsequently, 100 nM STX (not shown) completely blocked I_{Na} and again produced a suppression of gating charge movement, Q_{ON} = 10 nC/cm^2. Increasing the dose as high as 500 nM STX produced no additional suppression of I_gON above that seen with 10 nM STX. These data demonstrate that the degree of gating current suppression induced by external STX parallels the fraction of sodium channels blocked by toxin, and that increasing the dosage above that required to block all channels does not produce an additional effect on charge movement.

Internal Perfusion With TMA^+

Accurate measurement of I_gON in the absence of external toxin can only be achieved at precisely E_{Rev}. The presence of any sodium current contamination of I_gON would render kinetic comparisons of doubtful value, and prevent quantification of integrated gating charge movement. The possibility of ionic current contamination can be substan-

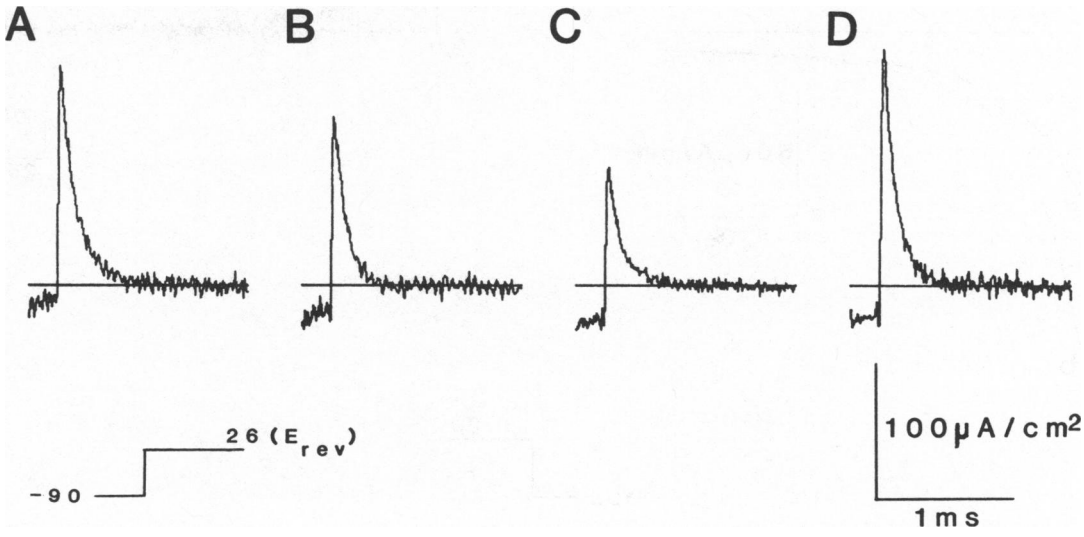

FIGURE 2 External STX reversibly suppresses gating current. Control I_gON (*A*) is compared with I_gON in 1 nM (*B*) and 10 nM external STX (*C*). Recovery of I_gON following washout of STX is shown in *D*. Membrane currents were measured at E_{Rev} (+26 mV), from a holding potential of −90 mV. Baseline represents zero current, with nonlinear leakage current visible below this baseline. Axon 043084, 25 Na MVH//230 Cs.

tially reduced by substituting impermeant ions for the permeant ions present in both the internal and external solutions. This substitution also permits the measurement of I_gON over a wider range of test potentials. Since tetramethylammonium (TMA^+) is known to be essentially impermeant through sodium channels (Hille, 1971), total replacement of all monovalent cations in both the internal and external solutions with TMA^+ was investigated as a method of eliminating ionic current contamination of I_gON records. However, intracellular TMA^+ has been reported to interfere with the inactivation of sodium channels in squid axons (Oxford and Yeh, 1985) and retard gating charge movement in *Myxicola* axons (Schauf, 1983). For these reasons, we have carefully evaluated the pharmacological effects of internal TMA^+ on sodium channel kinetics in crayfish axons.

In crayfish axons, substitution of 230 TMA for 230 Cs internal perfusate reduces peak I_{Na} (Fig. 3 *A*) but does not retard or suppress gating charge movement (Fig. 3 *B*). The suppression of I_{Na} is not accompanied by any significant change in the kinetics of either the rising or falling phase of sodium current, and therefore differs from the actions of TMA^+ in squid and *Myxicola* axons. Similar to other axon preparations, the reduction of I_{Na} by TMA^+ is dependent on test potential, as is illustrated in Fig. 4 *A*. Suppression of I_{Na} is minimal in the voltage range -50 to -30 mV, but increases at more depolarized potentials. This suppression of I_{Na} reaches an equilibrium value of ~50% at positive test potentials.

In Fig. 4 *B*, the effect on steady state inactivation of substituting 50 Na 180 TMA for 50 Na 180 Cs internal perfusate was determined by measuring I_{Na} at +20 mV after shifts of holding potential for a minimum of 3 min. The steady state inactivation curve is not affected by this substitution in the range of holding potentials -140 to -90 mV. Thus, in crayfish axons, internal TMA^+ produces a tonic block of I_{Na} at depolarized test potentials, but does not block sodium channels at rest and does not alter the steady state level of inactivation.

Although internal perfusion with TMA^+ does not alter sodium channel kinetics in crayfish axons, it remains necessary to demonstrate that this substitution can eliminate ionic current contamination of I_gON. As will be shown in the following sections, gating charge movement is not significantly affected by external STX or TTX at sufficiently hyperpolarized holding potentials. Therefore,

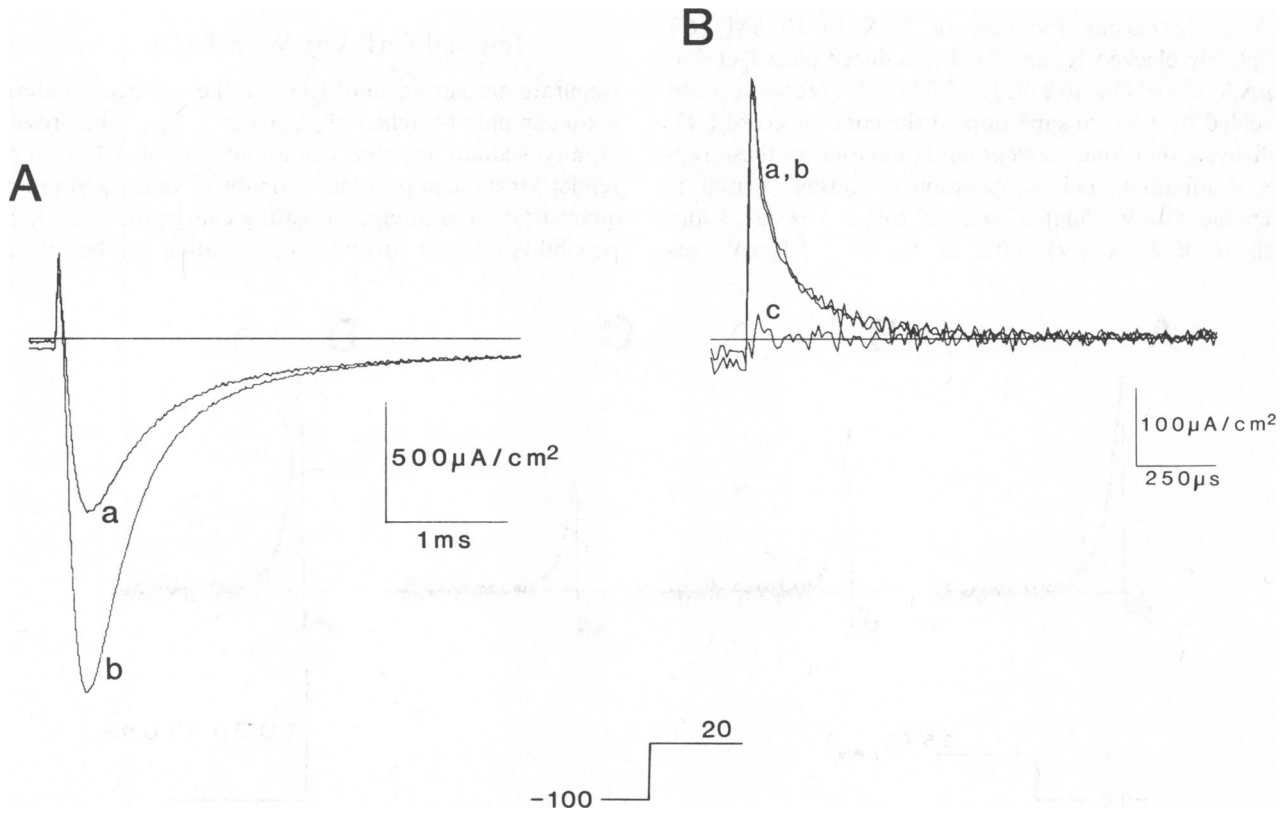

FIGURE 3 Substitution of TMA^+ for Cs^+ as the intracellular cation produces a suppression of I_{Na} but does not alter the kinetics of either I_{Na} or I_gON. In *A*, I_{Na} at +20 mV following substitution of 230 TMA (*a*) for 230 Cs (*b*) internal perfusate. In *B*, I_gON at +20 mV in the presence of 100 nM TTX, following substitution of 230 TMA (*a*) for 230 Cs (*b*). The difference record (*c*) generated by subtraction of *b* from *a* superimposes the zero current baseline. Holding potential −100 mV. *A*, Axon 052783, 50 Na MVH//230 Cs and 50 Na MVH//230 TMA; *B*, Axon 042384, 0 Na MVH//230 Cs and 0 Na MVH//230 TMA.

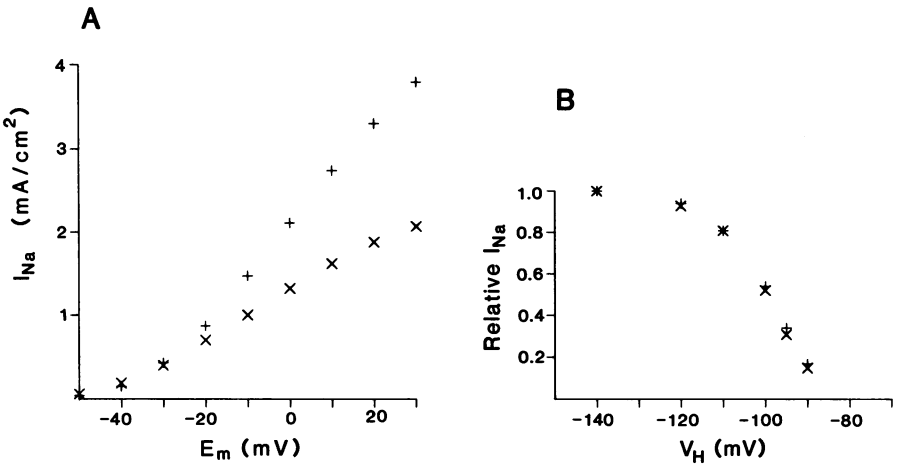

FIGURE 4 Internal perfusion with TMA^+ results in a voltage dependent suppression of I_{Na}, but does not alter the level of steady state inactivation. (*A*) comparison of the peak I_{Na} vs. V_m relationship between 230 Cs (+) and 230 TMA (X) internal perfusates. (*B*) steady state inactivation as measured from normalized peak I_{Na} +20 mV as a function of holding potential in Cs^+ (+) and TMA^+ (X) perfusates. Axon 011185, 0 Na MVH//50 Na 180 Cs and 0 Na MVH//50 Na 180 TMA.

using a holding potential of −130 mV, the presence or absence of ionic current contamination can be evaluated by a direct comparison between records obtained before and after addition of toxin. In Fig. 5, I_gON is compared before and after external application of 200 nM TTX, at three different test potentials: −20, +20 and +60 mV. At a test potential of −20 mV (*A*), a small inward ionic current is detectable. Armstrong and Bezanilla (1974) observed a similar ionic current at 0 mV in their experiments on squid axons and suggested that this current might reflect a small Ca^{++} flux through open sodium channels. At test potentials of +20 (*B*) and +60 mV (*C*), no ionic current is detectable, and the TTX records superimpose the control records collected in the absence of external toxin. These data demonstrate that this combination of internal and external TMA^+ solutions eliminates ionic current contamination of I_gON over the range +20 to +60 mV, without altering either the magnitude or kinetics of gating charge movement at these potentials.

In summary, internal substitution of 230 TMA for 230 Cs results in a tonic block of sodium channels in crayfish axons which: (1) is highly dependent on test potential, (2) does not significantly interfere with the inactivation of sodium channels, (3) does not suppress or retard gating charge movement, and (4) produces no shift in steady state inactivation as a function of membrane holding potential. We conclude that internal perfusion with TMA^+ coupled with a 0 Na^+ (TMA^+ substituted) external solution allows

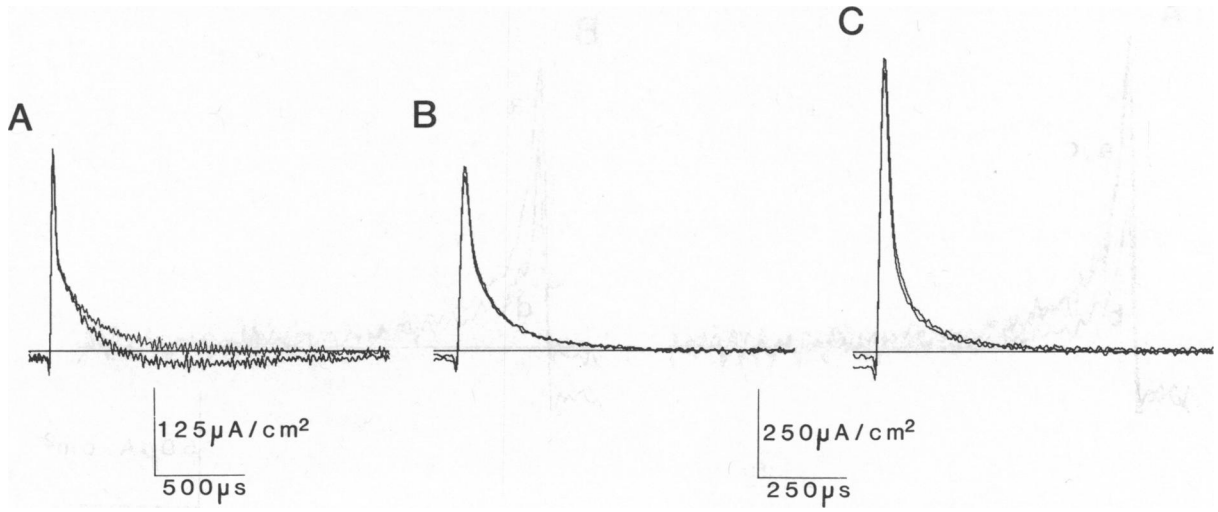

FIGURE 5 Accurate measurement of I_gON in the absence of external toxin is possible at positive test potentials when using 230 TMA internal perfusate. Records show membrane current before and after external application of 200 nM TTX at three different test potentials; (*A*) −20, (*B*) +20, and (*C*) +60 mV. Notice the presence of inward ionic current contamination in *A*, before external toxin. Holding potential −130 mV. Axon 022885, 0 Na MVH//230 TMA.

for accurate measurement of I_gON in the absence of external toxin. Based on these observations, many of the following experiments were performed with TMA^+ as the primary monovalent cation in both the internal and external solutions.

I_gON Kinetics Are Not Affected by STX

In Fig. 6 *A*, gating currents in 230 TMA are compared before (trace *a*) and during (trace *b*) treatment with external 100 nM STX. In these records with $V_H = -90$ mV, Q_{ON} at +20 mV has been reduced 57%, from 14 to 6 nC/cm^2, by the addition of 100 nM STX to the external solution. The suppressed I_gON in the presence of STX is kinetically indistinguishable from the control record taken before toxin treatment, and can be scaled by 2.3× (trace *c*) to superimpose the control record. This observation suggests that total charge movement is not simply retarded by the action of STX, but has indeed been reduced. The difference record (trace *d*) generated by direct subtraction of the STX record from the control is shown in Fig. 6 *B*. This difference record not only confirms that charge movement is suppressed by STX without kinetic distortion, but also demonstrates that ionic current is negligible under these experimental conditions.

We have investigated the possibility that this observed suppression of I_gON may result from an interaction between our intracellular perfusates and external STX. Synergistic interactions have previously been observed between internally applied organic cations capable of blocking sodium channel conductance and external TTX (Cahalan and Almers, 1979a, b; Armstrong and Croop, 1982). Gating currents are unaffected by these blocking cations in the absence of external toxin, but are suppressed by these agents in the presence of TTX. To exclude the possibility that internal Cs^+ or TMA^+ may produce a similar effect, we have measured I_gON at E_{Rev} with perfusates that contain Na^+ or K^+ as the sole internal cation. For these experiments, potassium channels were blocked by external application of 2 mM 4-aminopyridine. In both Na^+ and K^+ perfusates, we find that at holding potentials less negative than −100 mV, I_gON is substantially reduced in the presence of external STX or TTX. In other experiments, this suppression of I_gON by external toxin has also been observed in intact axons (no internal perfusion). Since this suppression of I_gON occurs under all these various experimental conditions, we conclude that external toxin exerts an effect on sodium channel gating that is independent of the intracellular monovalent cations.

Suppression of I_gON is Dependent on Holding Potential

We have tested whether the suppression of I_gON in STX is voltage dependent, and find no effect of test potential on the degree of suppression observed in the range +20 to +60 mV (not shown). However, this range of test potentials is in the plateau region of the Q_{ON} vs. V_m relationship in crayfish axon (Starkus et al., 1981), and a small voltage shift (i.e., 5–10 mV) might not produce a significant effect on charge movement. It would be more appropriate to look for an effect of test potential in the more voltage sensitive regions of the Q_{ON} vs. V_H plot (−50 to 0 mV), unfortunately, a detailed analysis of Q_{ON} in this range is prohibited by the presence of ionic current contamination at these voltages in the absence of external toxin (see Fig. 5 *A*).

Although the magnitude of gating current suppression

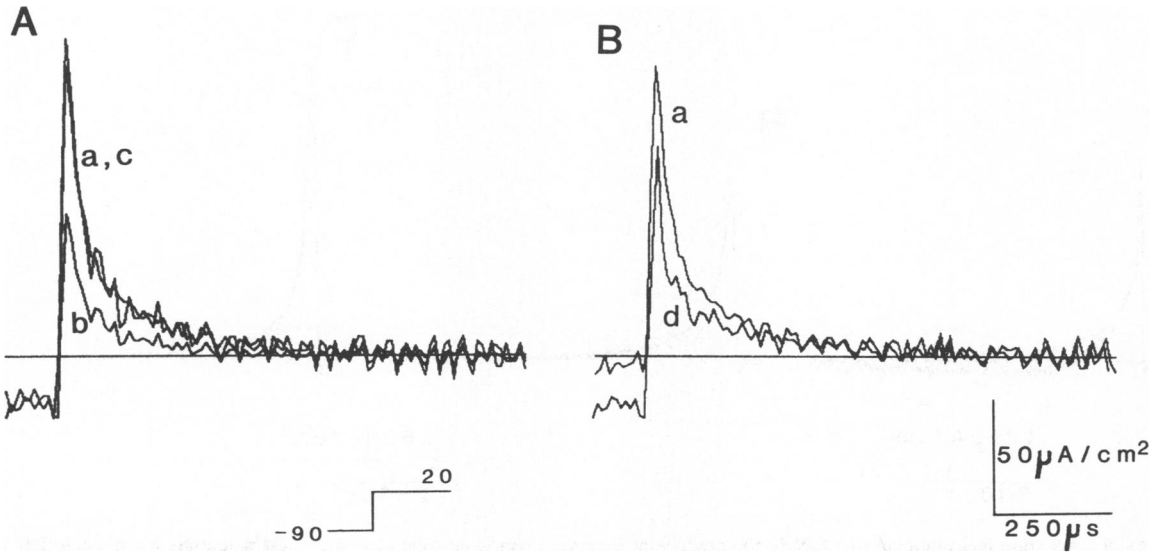

FIGURE 6 Total gating charge movement is suppressed by external STX at depolarized holding potentials, without altering the kinetics of I_gON. In *A*, control I_gON (no external toxin) at +20 mV (*a*), and in 100 nM STX (*b*). Gating current suppressed by external STX can be scaled by 2.3× (*c*) to superimpose the control record. In *B*, the difference record (*d*) was obtained by direct subtraction of *b* from *a*. Holding potential −90 mV. Axon 052584, 0 Na MVH//230 TMA.

by external STX is not dependent on test potential, it is highly dependent on holding potential. Fig. 7 compares I_gON at +20 mV, with three different holding potentials, before (*left column*) and during 100 nM STX application (*right column*). As holding potential is depolarized from −130 to −95 and then to −85 mV, steady state inactivation increases and the gating currents decrease. A comparison of the control I_gON with I_gON collected in 100 nM external STX clearly shows that the additional suppression of I_gON induced by external toxin is hold-potential dependent. In the top row of Fig. 7, with $V_H = -130$ mV, Q_{ON} is reduced by 5% after the addition of toxin, from 37 to 35 nC/cm^2. In the middle row, with V_H lowered to −95 mV, Q_{ON} was reduced by 41%, from 27 to 16 nC/cm^2, by the addition of 100 nM STX to the external solution. This large suppression of I_gON is not a result of loss of viable sodium channels (rundown), as nearly all of the charge movement was recovered upon returning to $V_H = -130$ mV. In the bottom row, at a holding potential of −85 mV, Q_{ON} is reduced from a control value of 9 nC/cm^2 to near zero in external 100 nM STX. These results demonstrate that as holding potential is made less negative and the steady state level of inactivation increases, the effect of STX in suppressing I_gON becomes more pronounced.

STX Shifts Steady State Inactivation

To further evaluate the relationship between holding potential and the toxin induced suppression of I_gON, Fig. 8 *A* compares Q_{ON} at +20 mV as holding potential is varied, both before and after external treatment with 200 nM STX. The suppression of I_gON is most pronounced in the range where the control Q_{ON} vs. V_H plot is steepest (−100 to −85 mV). In many axons, a small (5–15%) suppression of Q_{Max} is also observed, even at the most hyperpolarized hold potentials ($V_H = -140$ to −120 mV). This decrease in Q_{Max} may be due to a direct effect of toxin on charge movement that is independent of membrane potential. However, due to the absence of this suppression in some axons, and its variability when present, this small decrease in Q_{Max} probably results from a partial rundown of viable sodium channels during the 15–30 minute interval required to change solutions and stabilize temperature. To correct for rundown and permit a comparison with

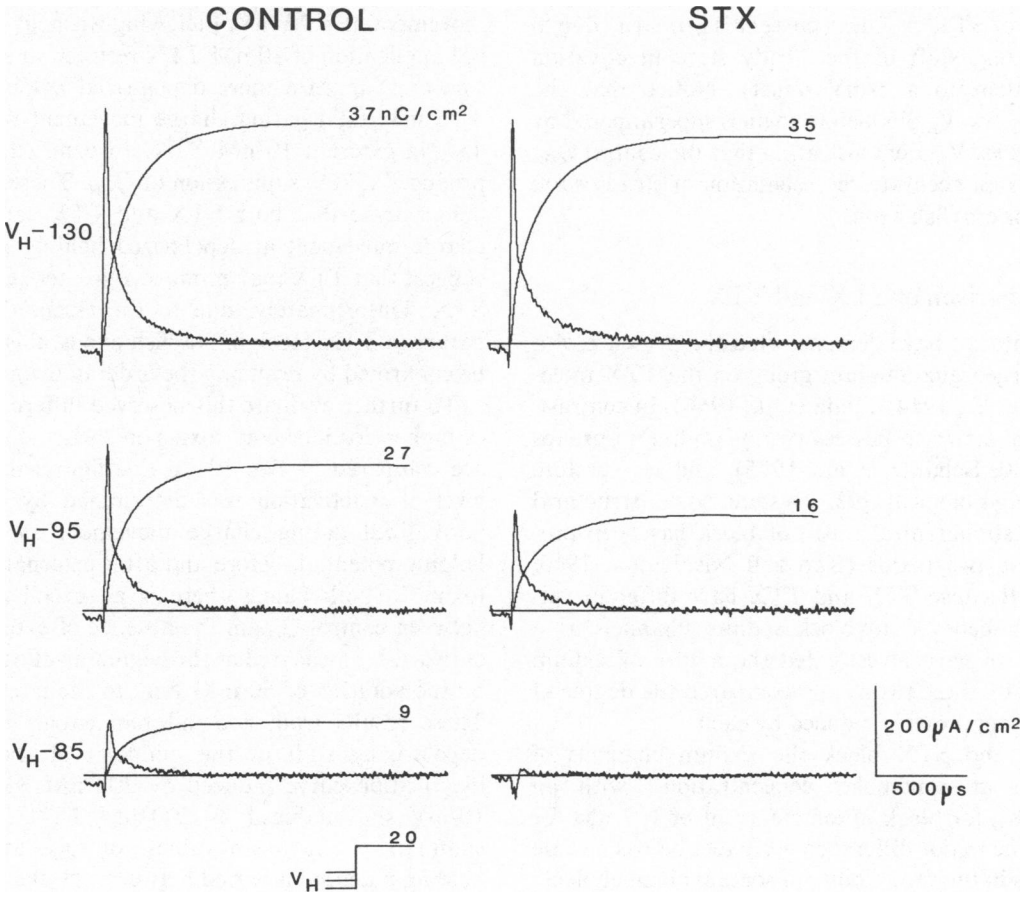

FIGURE 7 The suppression of I_gON by external STX is affected by holding potential. Gating currents at +20 mV and their integrations recorded without external STX (*left column*) are compared at three different holding potentials with similar records obtained in the presence of 100 nM STX (*right column*). Numbers adjacent to each integration record indicate total charge moved in nC/cm^2. Holding potential was varied from −130 mV (*top row*) to −95 mV (*middle row*), and −85 mV (*bottom row*). Axon 122884, 0 Na MVH//230 TMA.

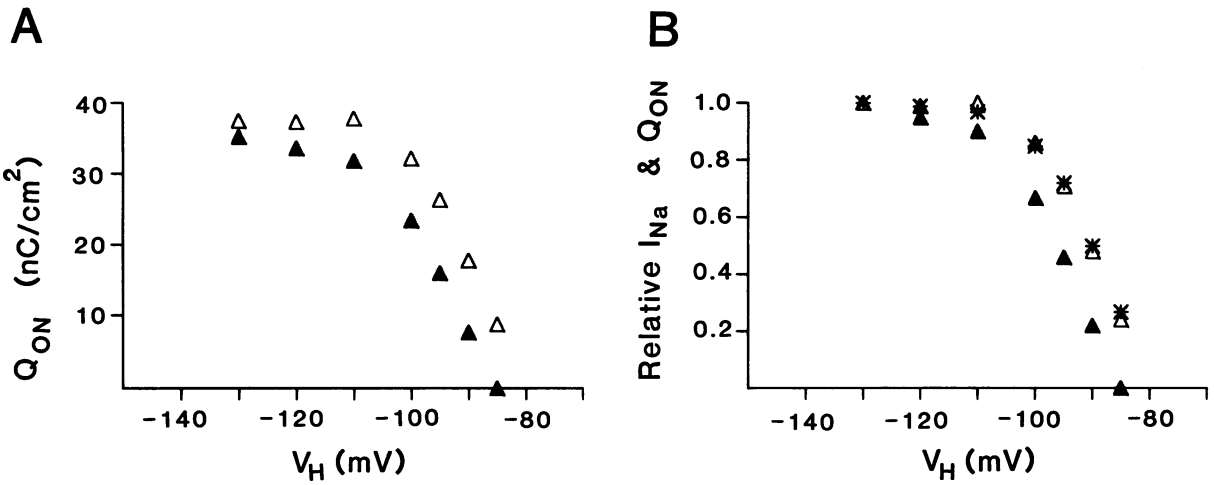

FIGURE 8 External STX shifts the steady state inactivation curve along the voltage axis. In *A*, Q_{ON} at +20 mV as holding potential is varied between −130 and −85 mV, before (△) and during (▲) exposure to external 200 nM STX. In *B*, the data from panel *A* are normalized, and compared with normalized I_{Na} (*) at +20 mV, collected with 50 mM Na added to the internal solution prior to STX. Axon 122884, 0 Na MVH//230 TMA (Q_{ON}) and 0 Na MVH//50 Na 180 TMA (I_{Na}).

steady state inactivation as measured by I_{Na}, Figure 8 *B* compares normalized Q_{ON} before and after external 100 nM STX with normalized I_{Na} measured with 50 mM Na^+ added to the internal perfusate. It appears from this plot that the effect of STX on gating current is primarily due to a hyperpolarizing shift of the steady state inactivation curve (equivalent to a 5-mV offset). Notice that the normalized Q_{ON} vs. V_H plot before toxin is superimposed by normalized I_{Na} vs. V_H, demonstrating that the control Q_{ON} vs. V_H curve is an accurate representation of steady state inactivation for crayfish axons.

Comparison of STX and TTX

Several laboratories have demonstrated the presence of a positively charged guanidinium group on the TTX molecule (Mosher et al., 1964; Tsuda et al., 1964). In contrast, STX has been shown to possess two guanidinium groups (Schantz, 1960; Schantz et al., 1975), and is therefore divalent at physiological pH. Despite some structural differences, a similar mechanism of block has been proposed for these two toxins (Kao and Nishiyama, 1965; Hille, 1975). Because STX and TTX have different valences, but are believed to block sodium channels at a common site, we have investigated the nature of sodium channel block by these toxins and compared the degree of gating current suppression induced by each.

Both TTX and STX block the sodium channels of crayfish axon at nanomolar concentrations, with an approximate K_D for block of ionic current of 1–2 nM for both toxins. The major difference we detect between these two agents lies in the reversibility of sodium channel block. At low doses, i.e. 10 nM, STX block is readily reversible on washout of external toxin, with a >75% recovery of I_{Na} occurring within 30 min. Block by TTX shows <10% recovery of I_{Na} after 30 min of toxin washout.

The effects of STX and TTX on I_gON are compared at two different holding potentials in Fig. 9. At a hyperpolarized holding potential of −120 mV, external application of 10 nM STX did not significantly suppress gating charge movement at +20 mV. Following washout of STX, external application of 10 nM TTX resulted in a 15% suppression of Q_{ON}. At a more depolarized holding potential of −95 mV, total gating charge movement was reduced by 45% in external 10 nM STX. In contrast, 10 nM TTX produced a 76% suppression of Q_{ON}. These results clearly demonstrate that both STX and TTX can affect gating charge movement at depolarized holding potentials, and suggest that TTX may produce a greater suppression than STX. Unfortunately, due to the essentially irreversible nature of TTX block in crayfish axons, this result can not be confirmed by reversing the order of drug applications.

To further evaluate this observed difference, the effects of higher doses of both toxins on steady state inactivation are compared in Fig. 10. In this figure, the steady state level of inactivation was determined by comparison of normalized gating charge movement as a function of holding potential, before and after external application of toxin. In both panels there is an excellent relationship between control Q_{ON} in the absence of external toxin and outward I_{Na} measured at the beginning of each experiment by the addition of 50 mM Na^+ to the internal perfusate. These results from two different axons reveal a 5 mV depolarizing shift of the midpoint of the steady state inactivation curve induced by 200 nM STX (*A*) and a 10-mV shift induced by 200 nM TTX (*B*). Table III summarizes the mean values for Q_{ON} at +20 mV as holding potential is varied between −140 and −70 mV; in the absence of toxin, in 200 nM STX and in 200 nM TTX. These data confirm that 200 nM TTX induces a greater shift of steady state inactivation than does 200 nM STX. A comparison of these results with data from low doses of

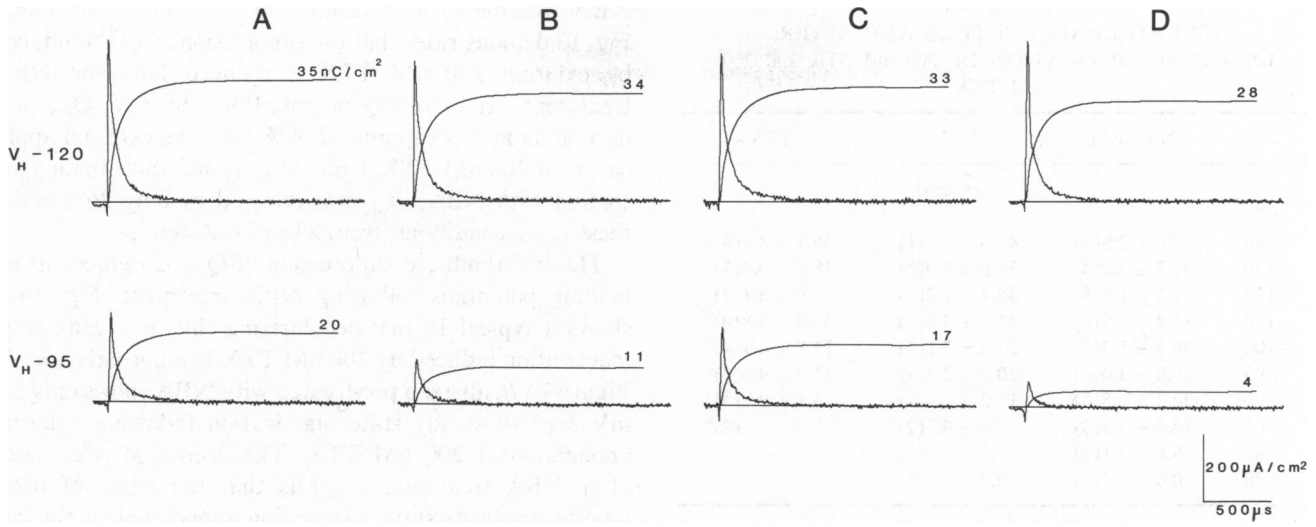

FIGURE 9 TTX produces a greater suppression of I_gON than STX. I_gON at +20 mV with integrated gating charge movement before toxin (*A*), in 10 nM STX (*B*), after washout of toxin (*C*), and in 10 nM TTX (*D*). Holding potential was varied from −120 mV (*top row*) to −90 mV (*bottom row*). Axon 010485, 0 Na MVH//230 TMA.

toxin (not shown) demonstrates that increasing the dose of toxin above that needed to produce a complete block of I_{Na} results in no additional shift in steady state inactivation. A mean shift in the steady state inactivation curve of 5.5 ± 2.1 mV (SEM n = 2) in 10 nM STX compares with 4.9 ± 3.0 mV (n = 3) in 200 nM STX. A greater shift is consistently observed in TTX, with steady state inactivation shifted by 8.5 ± 3.5 mV (n = 2) in 10 nM TTX and 10.0 ± 2.9 mV (n = 4) in 200 nM TTX.

Removal of Fast Inactivation

The results presented above suggest that both toxins suppress gating current indirectly, by shifting the voltage sensitivity of steady state inactivation. To further assess this relationship between steady state inactivation and the toxin induced suppression of I_gON, we have evaluated this phenomenon following both NBA and pronase pre-treatment. Internal perfusion with either pronase (Armstrong et al., 1973; Rojas and Rudy, 1976) or NBA (Oxford et al., 1978) is known to remove the fast inactivation of I_{Na} in squid axons, however, slow inactivation of I_{Na} is pronase resistant in squid axons (Rudy, 1978). If fast and slow inactivation can be enzymatically separated in crayfish axons, it may be possible to identify which component(s) are responsible for the shift of steady state inactivation induced by toxin binding.

In crayfish axons, before NBA treatment (see Fig. 11 *A*), I_{Na} at +20 mV inactivates rapidly via the fast inactivation mechanism. In this same axon, after a brief exposure to 1 mM NBA, fast inactivation is markedly reduced and I_{Na} at +20 mV fails to decay normally (see Fig. 11 *B*). Although fast inactivation has been suppressed by this treatment, slow, NBA resistant, component(s) of inactivation remain that are capable of inactivating sodium channels following depolarizing shifts of the holding poten-

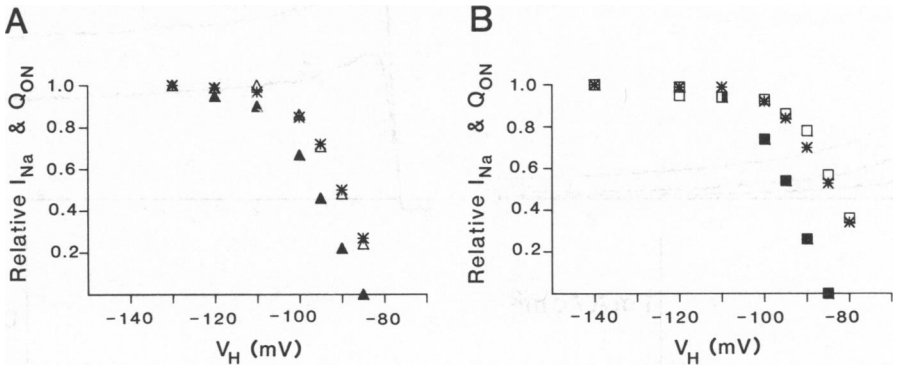

FIGURE 10 The effects of TTX on the steady state inactivation curve are similar to, but quantitatively greater than, the effects of STX. In *A*, normalized I_{Na} at +20 mV (*) is compared as a function of holding potential with normalized Q_{ON} at +20 mV before (△) and after external treatment with 200 nM STX (▲). In *B*, this same comparison is made between I_{Na} (*), control Q_{ON} (□), and Q_{ON} in 200 nM TTX (■). I_{Na} was measured before toxin by adding 50 mM Na^+ internally. (*A*) Axon 122884, 0 Na MVH//230 TMA (Q_{ON}) and 0 Na MVH//50 Na 180 TMA (I_{Na}); (*B*) Axon 122784, 0 Na MVH//230 TMA (Q_{ON}) and 0 Na MVH//50 Na 180 TMA (I_{Na}).

TABLE III
GATING CHARGE BEFORE AND AFTER EXTERNAL APPLICATION OF 200 nM STX and 200 nM TTX

V_h	No Toxin	STX	TTX
		nC/cm^2	
mV			
−140	40.6 ± 2.9(5)	41.0 (1)	35.7 ± 3.5(4)
−130	38.7 ± 3.0(4)	36.0 ± 2.0(3)	38.0 ± 4.5(3)
−120	38.6 ± 1.9(6)	35.4 ± 1.3(4)	34.0 ± 3.1(4)
−110	35.8 ± 1.9(6)	31.2 ± 2.3(3)	28.2 ± 3.8(4)[c]
−100	30.3 ± 1.7(6)	23.4 ± 2.1(4)[b]	19.2 ± 4.1(4)[b]
−95	26.0 ± 3.0(4)	20.2 ± 2.7(2)	11.7 ± 4.6(3)[b]
−90	20.4 ± 1.8(6)	11.2 ± 1.9(4)[b]	6.1 ± 2.7(4)[a]
−85	14.4 ± 3.3(3)	4.8 ± 4.7(2)[c]	0.0 (2)[b]
−80	8.6 ± 2.1(3)	0.5 (1)	—
−70	0.6 (1)	0.0 (1)	—

Data are given as mean ± SEM (n equals the number of axons). Probability of difference from control: [a]$p < .01$; [b]$p < .05$; [c]$p < .1$ all others show no significant difference ($p > .1$). Gating currents obtained with 0 Na MVH // 230 TMA.

tial. Figure 12 *A* compares normalized peak I_{Na} at +20 mV from this axon, as a function of holding potential, before and after NBA treatment. On another axon (Fig. 12 *B*), this same comparison is made from normalized control Q_{ON} at +20 mV. It is clear from these data that NBA treatment removes the fast inactivation of I_{Na} and also shifts the steady state inactivation curve (as determined from either ionic or gating current measurements) to more depolarized potentials.

To determine if external toxin can shift steady state inactivation and reduce I_gON following NBA treatment, we have compared the effects of TTX on normal axons (no NBA treatment) with results from NBA treated axons. Fig. 13 demonstrates that the suppression of I_gON induced by external 200 nM TTX is reduced following NBA treatment. At a holding potential of −90 mV, Q_{ON} in a normal axon was suppressed 50% after the external application of 200 nM TTX, from 24 to 12 nC/cm^2. In an axon treated with NBA, Q_{ON} was reduced by only 26% under these same conditions, from 23 to 17 nC/cm^2.

The toxin induced suppression of Q_{ON} is reduced at all holding potentials following NBA treatment. Fig. 14 *A* shows a typical 10 mV depolarizing shift in steady state inactivation induced by 200 nM TTX in a normal axon. In Figure 14 *B*, an axon pre-treated with NBA shows only a 3 mV shift of steady state inactivation following external application of 200 nM TTX. The decreased effect seen after NBA treatment suggests that the effect of toxin binding on steady state inactivation is mediated via the fast inactivation mechanism. One possible interpretation of these results is that TTX binding affects channel kinetics (prior to NBA) primarily by changing the voltage sensitivity of fast inactivation, while leaving slow inactivation unaltered. Since the removal of fast inactivation is incomplete at the NBA dosage used in these axons, it is possible that the small shift seen in these experiments may be related to the residual fast inactivation.

DISCUSSION

External STX and TTX Shift Steady State Inactivation

External application of either STX or TTX results in a suppression of gating currents in crayfish axons. The magnitude of this suppression is holding potential depen-

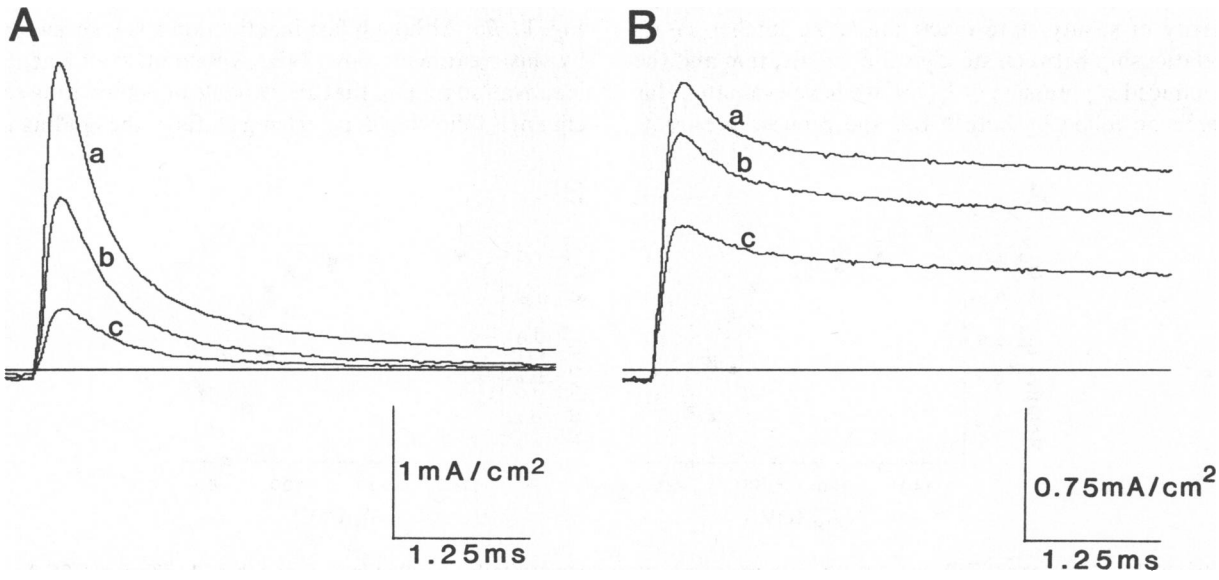

FIGURE 11 Internal treatment with NBA removes fast inactivation and decreases steady state inactivation at rest. In *A*, control I_{Na} at +20 mV as holding potential is varied from −120 mV (*a*) to −95 mV (*b*), and −85 mV (*c*). In *B*, I_{Na} at +20 mV after NBA treatment as holding potential is varied from −120 mV (*a*) to −95 mV (*b*), and −85 mV (*c*). Axon 011885, 0 Na MVH//50 Na 180 TMA.

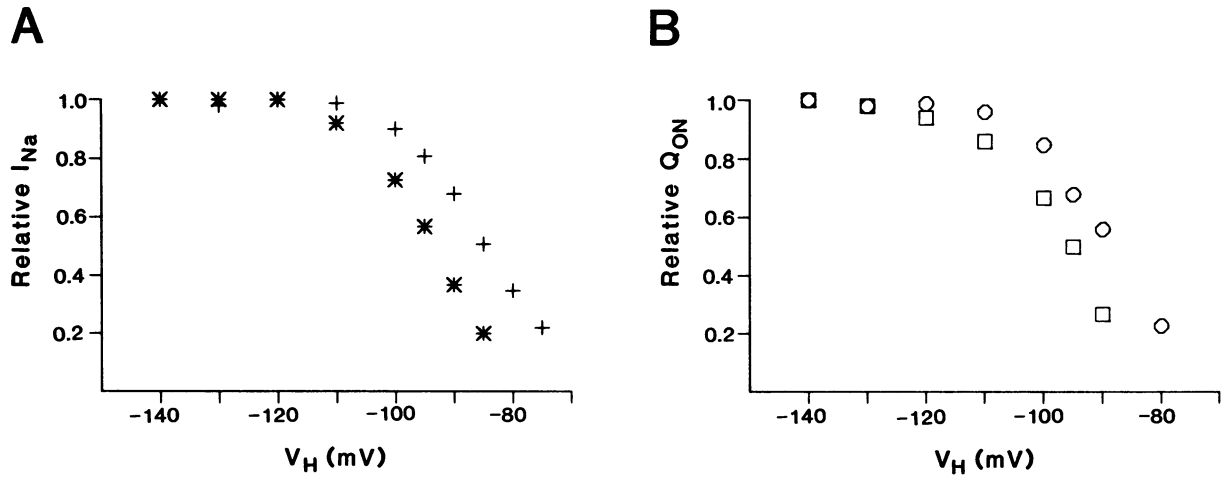

FIGURE 12 NBA treatment shifts the steady state inactivation of I_{Na} and Q_{ON}. In *A*, normalized peak I_{Na} at +20 mV as a function of holding potential in a normal axon (*) and following 5 min of internal perfusion with 1 mM NBA (+). In *B*, control Q_{ON} at +20 mV is plotted as a function of holding potential before (□) and after NBA treatment (○). (*A*) Axon 011885 0 Na MVH//50 Na 180 TMA (I_{Na}); (*B*) axon 011685 0 Na MVH//230 TMA (Q_{ON}).

dent and appears to reflect an increase in the level of steady state or resting inactivation. Although some suppression of gating charge movement occurs at all holding potentials tested, the suppression is most apparent at holding potentials more depolarized than −100 mV, where the level of steady state inactivation of sodium channels is most voltage sensitive. At these holding potentials, any small modification in the parameters of either activation or inactivation may affect large changes in channel availability.

Because the effect of external toxin on steady state inactivation is approximated by a 5–10 mV hyperpolarizing shift of the Q_{ON} vs. V_H plot (see Fig. 9), one possible

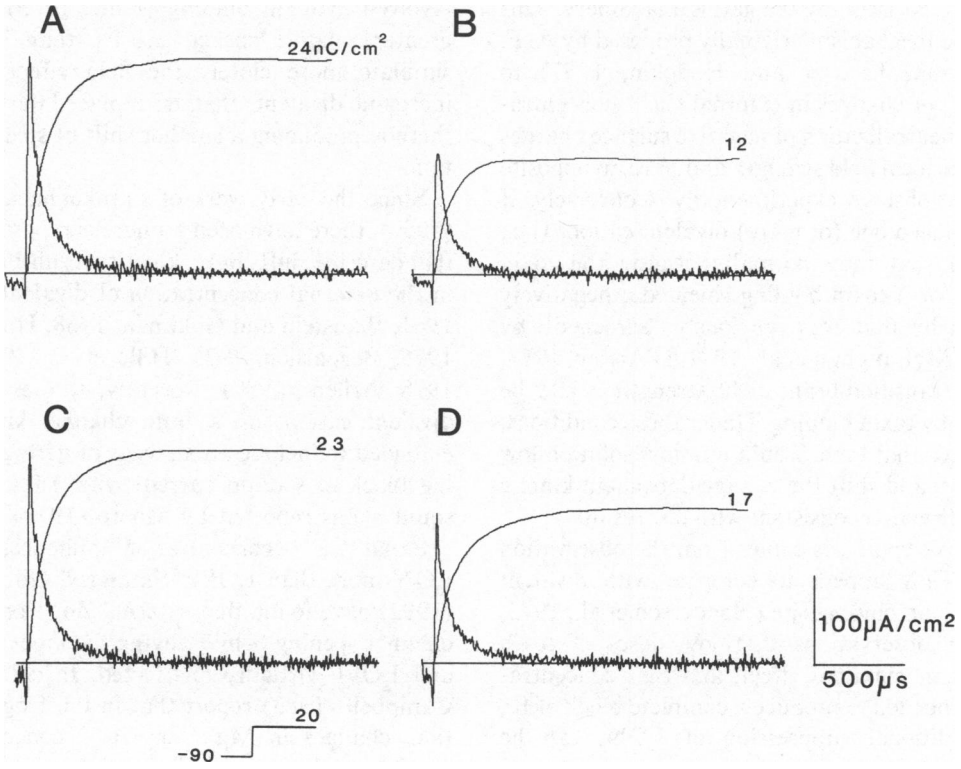

FIGURE 13 Internal NBA treatment partially removes the suppression of I_gON induced by external TTX. Data from normal axon (*top row*) shows control Q_{ON} at +20 mV (*A*) and following external 200 nM TTX (*B*). Data from NBA treated axon (*bottom row*) shows Q_{ON} at +20 mV before (*C*) and after (*D*) application of 200 nM TTX. All panels show both I_gON and integrated gating charge movement. (*A*) and (*B*) Axon 010985, 0 Na MVH//230 TMA; (*C*) and (*D*) axon 011685, 0 Na MVH//230 TMA.

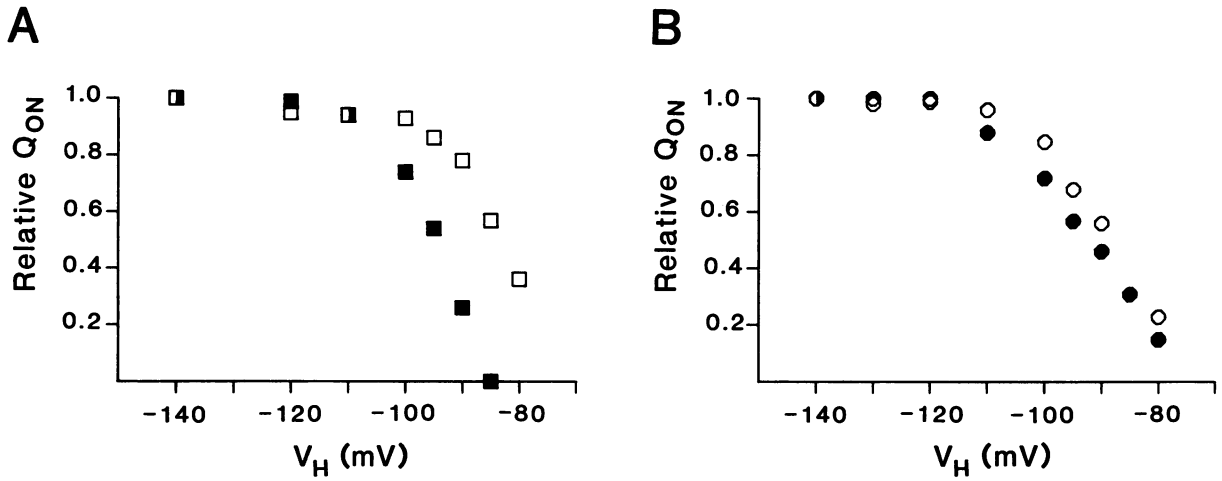

FIGURE 14 The effect of TTX on steady state inactivation is reduced after NBA treatment. In normal axon (*A*), normalized Q_{ON} at +20 mV is plotted as a function of holding potential before (□) and after (■) 200 nM external TTX. In an axon pretreated with NBA (*B*), normalized Q_{ON} at +20 mV is again plotted as a function of holding potential before (○) and after (●) 200 nM TTX. (*A*) Axon 122784, 0 Na MVH//230 TMA; (*B*) axon 011685 0 Na MVH//230 TMA.

interpretation of these results is that toxin binding may locally alter the transmembrane field in the region of the sodium channel voltage sensor. Both STX and TTX are positively charged at physiological pH, and therefore, toxin binding to the external portion of the sodium channel might be expected to partially offset fixed negative charges on the extracellular surface of the membrane, and thereby alter the potential "sensed" by the gating machinery. This is analogous to the mechanism originally proposed by A. F. Huxley (see Frankenhaeuser and Hodgkin, 1957) to explain the effects of changes in external Ca^{++} concentration. However, a neutralization of negative surface charges would increase the local field strength and have an opposite effect to what we observe experimentally. Conversely, if toxin binding replaced one (or more) divalent cations (i.e., Mg^{++} or Ca^{++}) that may normally occupy the toxin binding site, and/or if toxin binding shielded a negatively charged site nearby that was previously "screened" by external cations (McLaughlin et al., 1971; D'Arrigo, 1973; 1978), then the transmembrane field strength would be decreased locally by toxin binding. Under these conditions, the presence of external toxin would mimic a solution low in divalent cations, and shift the voltage dependent kinetic parameters in a direction consistent with our results.

Support for this hypothesis comes from the observation that STX and TTX appear to compete with divalent cations for a common binding site (Henderson et al., 1973, 1974). Also, our observations that low doses of toxin produce a less than maximal effect, and that concentrations above those needed to produce a complete block of I_{Na} result in no additional suppression of I_gON, can be explained within the framework of this hypothesis. At low doses of toxin, two sub-populations of channels exist; blocked and unblocked. Only blocked channels would have their steady state inactivation kinetics influenced by toxin binding, while unblocked channels would display normal kinetics. In addition, the observation that STX produces a smaller voltage shift than TTX can also be explained. Unlike TTX, which is monovalent (Mosher et al., 1964), STX bears a second positively charged guanidinium group (Schantz, 1960; Schantz et al., 1975), somewhat distant from the guanidinium group proposed to be the active site involved in toxin binding (Hille, 1975). Since STX has greater relative charge density than TTX, STX may simulate more closely the field effect induced by the inorganic divalents that are replaced during toxin binding, thereby producing a smaller shift of steady state inactivation.

Since the early work of Frankenhaeuser and Hodgkin (1957), there have been numerous reports in the literature that describe shifts in I_{Na} kinetics resulting from alterations in the external concentration of divalent cations (Dodge, 1961; Blaustein and Goldman, 1968; Hille, 1968; Schauf, 1975; Begenisich, 1975; Hille et al., 1975*b*; Shoukimas, 1978; Arhem, 1980). Recently, studies on the effects of divalent cations on sodium channel kinetics have been extended to include an analysis of gating currents, following block of sodium current with TTX. Experiments on squid axons reported by Moore (1978) indicate that the external Ca^{++} concentration influences the kinetics of I_gON more than I_gOFF. Similarly, Gilly and Armstrong (1982) have found that external Zn^{++} preferentially slows channel opening, while leaving the kinetics of tail currents and I_gOFF virtually unaffected. In contrast, Hahin and Campbell (1983) report that in the frog muscle preparation, changes in Mg^{++} or Ca^{++} concentration produce nearly equal shifts in all parameters of sodium channel kinetics, including steady state inactivation. Interestingly, in the experiments of Gilly and Armstrong (1982), the effects of Zn^{++} were substantially reduced after the exter-

nal application of TTX. These results suggest that external diavalent cations may influence sodium channel kinetics in several ways, at least one of which is TTX sensitive.

We have looked for changes in the kinetics of channel opening following TTX treatment. However, a comparison of gating current kinetics before and after application of external toxin demonstrates that in the voltage range +20 to +60 mV, suppression of I_gON occurs without any observable kinetic differences. It should be pointed out that this range of potentials is at the plateau region of the Q_{ON} vs. V_m relationship (Starkus et al., 1981), where activation kinetics are not very voltage sensitive in crayfish axons, and a small (5–10 mV) shift of kinetics could easily go undetected. The kinetics of activation are most voltage sensitive in the range of test potentials −50 to 0 mV, however, ionic current contamination of I_gON in the absence of external toxin (see Fig. 5) prevents us from making accurate kinetic comparisons at these voltages.

Comparison With Squid Axons

In squid axon, no suppression of I_gON has been observed with external TTX (Armstrong and Bezanilla, 1974; Gilly and Armstrong, 1982) or with external STX (Keynes and Rojas, 1974). The conflicting results seen in these two preparations may result from differences in the voltage dependence of steady state inactivation. Fig. 15 shows the differences in the steady state inactivation curves for squid and crayfish axons, demonstrating that at V_H = −90 mV, a 5–10 mV shift will be considerably more noticeable in crayfish axons. With intact crayfish axons, we typically find an initial resting potential of −95 to −100 mV, which is near the top of the steady state inactivation curve. Whereas, in squid axons, typical resting potentials are reported to be much lower, in the range of −60 to −65 mV (Hodgkin and Huxley, 1952*a*).

Gating currents collected from crayfish axons in the plateau region of the Q_{ON} vs. V_H plot (−140 to −120 mV) show only minimal effects of external toxin binding, whereas records collected from holding potentials on the steepest portion of the plot (i.e., −100 to −70 mV for crayfish axons) show the maximal effect on charge movement. The effect of external toxins on steady state inactivation might also occur in squid axon preparations; however, because gating currents are typically recorded with holding potentials at the top of the steady state inactivation curve, these effects may not be readily apparent. Also, since the steady state inactivation curve is less steep in squid axons, any shift of steady state inactivation would result in a correspondingly smaller suppression of gating current. Suppression of I_gON by STX and TTX might be more apparent in squid axons if holding potentials were adjusted to the steepest regions of the steady state inactivation curve for squid.

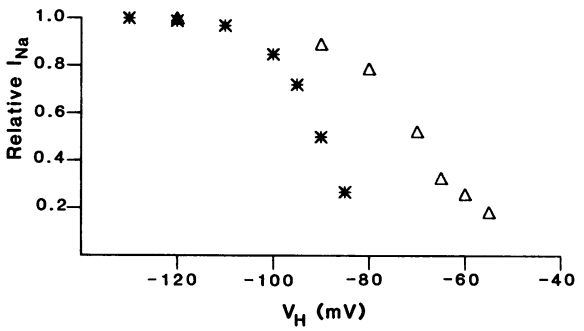

FIGURE 15 A comparison of steady state inactivation as a function of membrane potential in crayfish and squid axons demonstrates substantial differences in their voltage dependences. Data for squid axon (Δ) was adapted from 2 min prepulse experiments of Adelman and Palti (1969, Fig. 5). Typical data shown for crayfish axon (*) was collected by shifting V_H for a duration of 3 min (data from Fig. 8 *B*). Data shown is normalized I_{Na} as a function of holding potential.

Effects of NBA Treatment

Inactivation of the sodium conductance system is a complex process that is still only poorly understood. Sodium currents at depolarized test potentials are observed to decay in a voltage and time dependent fashion, originally described and modeled as a single exponential decay (Hodgkin and Huxley, 1952*b*). Subsequent studies have demonstrated that the process of sodium current inactivation is indeed more complicated than these authors originally proposed. Work by Chiu (1977) has shown that the inactivation of sodium current in the frog node preparation is not well fit by a single exponential, but rather is better modeled as the sum of two (or more) decaying exponentials. A recent analysis of the inactivation kinetics of crayfish axon sodium current (Rayner and Starkus, 1985) demonstrates multiple components on the falling phase of I_{Na}. Several laboratories (Adelman and Palti, 1969; Khodorov et al., 1976; Shrager, 1977) have shown that the re-equilibration of the sodium conductance system following various duration pre-pulses results from the interaction of both fast and slow inactivation processes. We have therefore attempted to resolve which of these separable components is affected by toxin binding.

Intracellular treatment with NBA removes the fast inactivation of I_{Na} that is seen with depolarizing test pulses (Oxford et al., 1978). In crayfish axons, we observe that most of the fast inactivation of I_{Na} is prevented following NBA, but that the slow component(s) of inactivation remain, and result in the establishment of a new steady state level of resting inactivation (see Fig. 12). The level of steady state inactivation is still holding potential sensitive after NBA treatment, but the curve is shifted along the voltage axis by ~10 mV in the depolarizing direction. Results that are qualitatively similar to these are also achieved in crayfish axons after the removal of fast inactivation by intracellular pronase treatment (unpublished observations). It is not clear from these data how the fast and slow components of inactivation interact to reach a

steady state level, only that these two processes can be separated enzymatically, and that such treatments influence the final equilibrium distribution of sodium channels at rest.

After NBA treatment, the suppression of I_gON by TTX is reduced (see Fig. 13). However, the data of Fig. 13 does not indicate whether the reduced effect of external toxin is due to a direct effect of NBA treatment, or results from a corresponding shift in steady state inactivation. This question is addressed in Fig. 14, where the steady state inactivation curve is compared before and after external application of 200 nM TTX. Clearly, following NBA, there is a reduced effect of external toxin across all holding potentials. This result seems to indicate that external toxin exerts its action on gating current through electrostatic effects that alter the voltage sensitivity of the fast (NBA sensitive) inactivation mechanism, while having little effect on slow inactivation. Alternatively, it is possible that NBA may remove the effect of external toxin by altering the channel architecture in some other way, unrelated to its actions on fast inactivation.

The authors thank Dr. Martin Rayner for helpful advice and comments on this manuscript. We also wish to thank Joe Risser and Cary Miyahara for their excellent technical assistance.

This work was performed with the support of the National Institutes of Health through the New Investigator Research Award NS17202 and research grant NS21151 (to John G. Starkus). Additional support was received from the Hawaii Heart Association and also in part from the University of Hawaii Research Council, and from BRSG S07 RR 0726.

Received for publication 24 August 1984 and in final form 10 October 1985.

REFERENCES

Adelman, W. J., and Y. Palti. 1969. The effects of external potassium and long duration voltage conditioning on the amplitude of sodium currents in the giant axon of the squid, Loligo pealei. *J. Gen. Physiol.* 54:589–606.

Arhem, P. 1980. Effects of some heavy metal ions on the ionic currents of myelinated fibers from Xenopus laevis. *J. Physiol. (Lond.).* 306:219–231.

Armstrong, C. M., and F. Bezanilla. 1974. Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. Gen. Physiol.* 63:533–552.

Armstrong, C. M., and R. S. Croop. 1982. Simulation of Na channel inactivation by thiazin dyes. *J. Gen. Physiol.* 80:641–662.

Armstrong, C. M., F. Bezanilla, and E. Rojas. 1973. Destruction of sodium conductance inactivation in squid axons perfused with pronase. *J. Gen. Physiol.* 62:375–391.

Begenisich, T. 1975. Magnitude and location of surface charges on *myxicola* giant axons. *J. Gen. Physiol* 66:47–65.

Blaustein, M. P., and D. E. Goldman. 1968. The action of certain polyvalent cations on the voltage-clamped lobster axon. *J. Gen. Physiol.* 51:279–291.

Cahalan, M. D., and W. Almers. 1979*a*. Interactions between quaternary lidocaine, the sodium channel gates, and tetrodotoxin. *Biophys. J.* 27:39–56.

Cahalan, M. D., and W. Almers. 1979*b*. Block of sodium conductance and gating current in squid giant axons poisoned with quaternary strychnine. *Biophys. J.* 27:57–74.

Chiu, S. Y. 1977. Inactivation of sodium channels: second order kinetics in myelinated nerve. *J. Physiol. (Lond.).* 273:573–596.

Cohen, C. J., B. P. Bean, T. J. Colatsky, and R. W. Tsien. 1981. Tetrodotoxin block of sodium channels in rabbit purkinje fibers: interactions between toxin binding and channel gating. *J. Gen. Physiol.* 78:383–411.

D'Arrigo, J. S. 1973. Possible screening of surface charges on crayfish axons by polyvalent metal ions. *J. Physiol. (Lond.).* 231:117–128.

D'Arrigo, J. S. 1978. Screening of membrane surface charges by divalent cations: an atomic representation. *Am. J. Physiol.* 235(3):C109–C117.

Dodge, F. A. 1961. Ionic permeability changes underlying nerve excitation. *In* Biophysics of Physiological and Pharmacological Actions. American Association for the Advancement of Science, Washington, DC 119–143.

Frankenhaeuser, B. and A. L. Hodgkin. 1957. The action of calcium on the electrical properties of squid axons. *J. Physiol. (Lond.).* 137:218–244.

Gilly, W. F., and C. M. Armstrong. 1982. Slowing of sodium channel opening kinetics in squid axon by extracellular zinc. *J. Gen. Physiol.* 79:935–964.

Henderson, R., J. M. Ritchie, and G. R. Strichartz. 1973. The binding of labelled saxitoxin to the sodium channels in nerve membranes. *J. Physiol. (Lond.).* 235:783–804.

Henderson, R., J. M. Ritchie, and G. R. Strichartz. 1974. Evidence that tetrodotoxin and saxitoxin act at a metal cation binding site in the sodium channel of nerve membrane. *Proc. Natl. Acad. Sci. USA.* 71:3936–3940.

Hahin, R., and D. T. Campbell. 1983. Simple shifts in the voltage dependence of sodium channel gating caused by divalent cations. *J. Gen. Physiol.* 82:785–802.

Hille, B. 1968. Charges and potentials at the nerve surface: divalent ions and pH. *J. Gen. Physiol.* 51:221–236.

Hille, B. 1971. The permeability of the sodium channel to organic cations in myelinated nerve. *J. Gen. Physiol.* 58:599–619.

Hille, B. 1975. The receptor for tetrodotoxin and saxitoxin: a structural hypothesis. *Biophys. J.* 15:615–619.

Hille, B., J. M. Ritchie, and G. S. Strichartz. 1975*a*. The effect of membrane surface charge on the action of tetrodotoxin and saxitoxin on frog myelinated nerve. *J. Physiol. (Lond.).* 250:34P–35P.

Hille, B., A. M. Woodhull, and B. I. Shapiro. 1975*b*. Negative surface charge near sodium channels of nerve: divalent ions, monovalent ions, and pH. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 270:301–318.

Hodgkin, A. L., and A. F. Huxley. 1952*a*. Currents carried by sodium and potassium ions through the membrane of the giant axon of loligo. *J. Physiol. (Lond.).* 116:449–472.

Hodgkin, A. L., and A. F. Huxley. 1952*b*. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol. (Lond.).* 117:500–544.

Kao, C. Y., and A. Nishiyama. 1965. Action of saxitoxin on peripheral neuromuscular systems. *J. Physiol. (Lond.).* 180:50–66.

Keynes, R. D., and E. Rojas. 1974. Kinetics and steady state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol. (Lond.).* 239:393–434.

Khodorov, B., L. Shishkova, E. Peganov, and S. Revenko. 1976. Inhibition of sodium currents in frog ranvier node treated with local anesthetics. Role of slow sodium inactivation. *Biochim. Biophys. Acta.* 433:409–435.

McLaughlin, S. G. A., G. Szabo, and G. Eisenman. 1971. Divalent ions and surface potential of charged phospholipid membranes. *J. Gen. Physiol.* 58:667–687.

Moore J. W. 1978. On sodium conductance gates in nerve membranes. *In* Physiology and Pathobiology of Axons. S. G. Waxman, editor. Raven Press, New York. 145–153.

Moore, J. W., and T. Narahashi. 1967. Tetrodotoxin's highly selective blockage of an ionic channel. *Fed. Proc.* 26:1655–1663.

Mosher, H. S., F. A. Fuhrman, H. D. Buchwald, and H. G. Fischer. 1964.

Tarichatoxin-tetrodotoxin: a potent neurotoxin. *Science.* (Wash. DC) 144:1100–1110.

Narahashi, T., J. W. Moore, and W. R. Scott. 1964. Tetrodotoxin blockage of sodium conductance increase in lobster giant axons. *J. Gen. Physiol.* 47:965–974.

Oxford, G. S., C. H. Wu, and T. Narahashi. 1978. Removal of sodium channel inactivation in squid axons by *N*-bromoacetamide. *J. Gen. Physiol.* 71:227–247.

Oxford, G. S., and J. Z. Yeh. 1985. Interactions of monovalent cations with sodium channels in squid axon. I. Modification of physiological inactivation gating. *J. Gen. Physiol.* 85:583–602.

Rayner, M. D., and J. G. Starkus. 1985. Multiple exponential components in the falling phase of sodium current. *Biophys. J.* 47(2, pt. 2):31*a*. (Abstr.)

Rojas, E., and B. Rudy. 1976. Destruction of the sodium conductance inactivation by a specific protease in perfused nerve fibers from loligo. *J. Physiol. (Lond.).* 262:502–531.

Rudy, B. 1978. Slow inactivation of the sodium conductance in squid giant axons pronase resistance. *J. Physiol. (Lond.).* 283:1–22.

Schantz, E. J. 1960. Biomedical studies of paralytic shellfish poisons. *Ann. NY Acad. Sci.* 90:843–855.

Schantz, V. E. Ghazarossian, H. K. Schnoes, F. M. Strong, J. P. Springer, J. O. Pezzanite, and J. Clardy. 1975. The structure of saxitoxin. *J. Am. Chem. Soc.* 97:1238–1239.

Schauf, C. L. 1975. The interactions of calcium with *Myxicol* giant axons and a description in terms of a simple surface charge model. *J. Physiol. (Lond.).* 248:613–624.

Schauf, C. L. 1983. Tetramethylammonium ions alter sodium-channel gating in *myxicola. Biophys. J.* 41:269–274.

Shoukimas, J. J. 1978. Effect of calcium upon sodium inactivation in the giant axon of Loligo pealei. *J. Membr. Biol.* 38:271–289.

Shrager, P. 1974. Ionic conductance changes in voltage clamped crayfish axons at low pH. *J. Gen. Physiol.* 64:666–690.

Shrager, P. 1977. Slow sodium inactivation in nerve after exposure to sulfhydryl blocking reagents. *J. Gen. Physiol.* 69:183–202.

Starkus, J. G., B. D. Fellmeth, and M. D. Rayner. 1981. Gating currents in the intact crayfish giant axon. *Biophys. J.* 35:521–533.

Starkus, J. G., and S. T. Heggeness. 1985. Suppression of sodium channel gating current by extracellular saxitoxin and tetrodotoxin in crayfish axons. *Biophys. J.* 47(2, pt. 2):32*a*. (Abstr.)

Starkus, J. G., S. T. Heggeness, and M. D. Rayner. 1984. Kinetic analysis of sodium channel block by internal methylene blue in pronased crayfish giant axons. *Biophys. J.* 46:205–218.

Starkus, J. G., and P. Shrager. 1978. Modification of slow sodium inactivation in nerve after internal perfusion with trypsin. *Am. J. Physiol.* 4:C238–244.

Tsuda, K., R. Tachikawa, K. Sakai, C. Tamura, O. Amakasu, M. Kawamura, and S. Ikuma. 1964. On the structure of tetrodotoxin. *Chem. Pharm. Bull. (Tokyo).* 12:642–645.

Van Harreveld, A. 1936. A physiological solution for fresh water crustaceans. *Proc. Soc. Exp. Biol. Med.* 34:428–432.