INTERACTIONS BETWEEN QUATERNARY LIDOCAINE, THE SODIUM CHANNEL GATES, AND TETRODOTOXIN

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ABSTRACT A voltage clamp technique was used to study sodium currents and gating currents in squid axons internally perfused with the membrane impermeant sodium channel blocker, QX-314. Block by QX-314 is strongly and reversibly enhanced if a train of depolarizing pulses precedes the measurement. The depolarization-induced block is antagonized by external sodium. This antagonism provides evidence that the blocking site for the drug lies inside the channel. Depolarization-induced block of sodium current by QX-314 is accompanied by nearly twofold reduction in gating charge movement. This reduction does not add to a depolarization-induced immobilization of gating charge normally present and believed to be associated with inactivation of sodium channels. Failure to act additively suggests that both, inactivation and QX-314, affect the same component of gating charge movement. Judged from gating current measurements, a drug-blocked channel is an inactivated channel. In the presence of external tetrodotoxin and internal QX-314, gating charge movement is always half its normal size regardless of conditioning, as if QX-314 is then permanently present in the channel.

INTRODUCTION

The voltage dependence or "gating" of sodium channels received its first and detailed description some time ago (Hodgkin and Huxley, 1952*b*), but progress in studying its molecular mechanism has been slow. New opportunities have arisen from the discovery of "gating currents" (Armstrong and Bezanilla, 1974), small and relatively slow capacitive currents that are now generally believed to arise from the molecular transitions undergone by sodium channels as they open and close (Armstrong and Bezanilla, 1974; Keynes and Rojas, 1974; Meves, 1974; Nonner et al., 1975). These gating currents are theoretically expected to arise whenever a change in membrane potential causes movement of the dipolar or charged molecular groups from which sodium channels derive their steep voltage dependence. Recent reviews of the relevant literature have appeared (Neumcke et al., 1978; Meves, 1978; Almers, 1978).

This paper and the next (Cahalan and Almers, 1979) deal with an effect called "charge immobilization" (Armstrong and Bezanilla, 1977): under maintained depolarization, the voltage-sensitive portions of sodium channels become partially immobilized so that they cannot produce full-sized gating currents during subsequent potential steps. Charge immobilization is thought to accompany inactivation of sodium channels; in a model by Armstrong and Bezanilla (1977), both events are caused by an endogenous, membrane-bound blocking

particle that enters the channel from the inside, interrupting ion flow and sterically hindering subsequent conformational changes.

Many small molecules (mol wt, 250–500) are now known to block sodium currents from the axoplasmic side, perhaps by inserting themselves into the channel. Among them are local anesthetics (Strichartz, 1973; Courtney, 1975; Hille, 1977, Cahalan, 1978), strychnine (Shapiro, 1977; Cahalan, 1978); pancuronium (Yeh and Narahashi, 1977), arginine (Eaton and Brodwick 1978), and, in pronase-treated axons, quaternary ammonium compounds (Rojas and Rudy, 1976). Do these artificial blocking particles cause charge immobilization when they block? In this and the following paper we investigate two of them, namely QX-314, a quaternary lidocaine derivative, and *N*-methylstrychnine. New and published data are summarized that strongly suggest that the binding site for both compounds lies within the channel. Both substances are found to cause charge immobilization when they block, and we compare this artificial charge immobilization with the physiological one. This paper deals with QX-314 only. Preliminary accounts of this work have been given (Almers and Cahalan, 1977; Almers, 1978).

METHODS

Giant axons from the squid, *Loligo pealii,* were studied under voltage clamp at the Marine Biological Laboratory in Woods Hole, Mass. The details of the internal perfusion and voltage clamp technique are found in Begenisich and Lynch (1975) (see also Cahalan and Begenisich, 1976) and are briefly described below. Carefully cleaned segments of squid giant axons, 25–30 mm long and 0.35–0.6 mm in diameter, were cannulated and perfused initially with standard internal solution (350 mM K^+-glutamate, 50 mM K^+-fluoride, 50 mM sucrose, 10 mM K-phosphate buffer of pH 7.3). 1 mg/ml papain (Sigma Chemical Co., St. Louis, Mo.) was added later to digest away most of the axoplasm in the air gap and thereby facilitate perfusion. Prolonged presence of papain would ultimately remove sodium inactivation, but in our experiments exposure of the central region of the axon to papain lasted < 1 min, insufficient to allow significant effects of this kind. Finally, the axon was voltage-clamped and perfusion switched to one of the internal solutions shown in Table I. Internal and external solutions (Table I) were exchanged at 3–5 vol/min during experiments. Throughout, solutions are indicated in figure legends as $x//y$ where x is the external and y the internal solution. The temperature was measured by a thermistor next to the axon and in most experiments was maintained at 8°C by a thermoelectric device. In some experiments the temperature was raised to 16°C to increase the amplitude of gating currents.

The membrane potential, V, was measured between an inner pipette filled with 0.56 M KCl and an

TABLE I
SOLUTIONS

	[Na]	[Cs]	[Ca]	[Glutamate]	[Mg]	[F]	[Tris]	[Cl]	[Sucrose]	
External					*mM*					*pH*
Na-ASW	440	—	10	—	50	—	10	560	—	7.4
Tris-ASW	—	—	10	—	50	—	450	570	—	7.4
x Na-ASW	x	—	10	—	50	—	$440 - x$	560	—	7.4
Internal										
200 Na	200	—	—	125	—	75	10	—	510	7.3
x Na	x	$200 - x$	—	125	—	75	10	—	510	7.3
200 Cs	0	200	—	100	—	100	10	—	510	7.3

Na-ASW, normal artificial sea water.

external sea water bridge, each connected to a silver-silver chloride electrode. The potential was controlled by electronic feedback, employing compensation for series resistance of 3 Ω-cm^2. The holding potential in these experiments was -70 mV. Membrane current was amplified and corrected in a preliminary way for leakage and capacitive transients, using analog circuitry. The signals were then either photographed off an oscilloscope (Figs. 1 and 2) or (in all other experiments) digitized at up to 100 kHz sampling frequency at 12-bit accuracy and stored on shift register buffers of a signal averager (Bezanilla and Armstrong, 1977). The signal averager allowed a final linear correction for capacitive and leakage admittances by recording and summing, for each "test pulse," membrane currents during four small "control pulses" of equal duration given at 0.125-s interval. Whereas test pulses started from -70 mV, control pulses started from a potential so negative (-130 mV unless otherwise specified) that they did not cause membrane depolarization beyond -70 mV. The "bias potential" of -130 mV was established 60 ms before and removed 8 ms after the end of each control pulse. The amplitude of each control pulse was exactly one-quarter that of the test pulse. Therefore when currents during the four control pulses were summed and subtracted from that during the test pulse, only the "asymmetry current" due to nonlinear membrane properties should remain (P/4 procedure, Armstrong and Bezanilla, 1977). The sequence of four control and one test pulse could be repeated for signal averaging.

The asymmetry current, or a signal-averaged version thereof, was stored on DEC-tape at maximally 9-bit resolution for later analysis on a PDP-8 (Digital Equipment Corporation, Marlboro, Mass.) or Raytheon-440 computer (Raytheon Data Systems Co., Norwood, Mass.). We thank Dr. Eberhard Fetz (PDP-8) and Dr. Theodore Kehl (Raytheon-440), both at the University of Washington, for the use of their facilities. Data analysis on the PDP-8 was performed with computer programs developed by Doctors Francisco Bezanilla, Clay M. Armstrong and Robert F. Rakowski. QX-314 was kindly provided by Dr. Bertil Takman of Astra Pharmaceuticals Products, Inc., Framingham, Mass.

RESULTS

Block of Sodium Currents by QX-314 and Antagonism by External Sodium

After internal application of 1 mM QX-314, sodium currents in response to depolarizing pulses diminish. At low stimulation frequencies (0.33 pulses/min) the drug reduced currents to 80 ± 5% SEM (n = 3, 0.44 M external sodium) or 43% ± 8% SEM (n = 3, 20–40 mM external sodium) of their normal value; pulses were 70 mV in amplitude and started from -70 mV, the holding potential used throughout. After drug application and withdrawal, this "tonic block" developed and reversed with a half-time of 1–5 min. Drug washout was not attempted routinely.

At higher stimulus frequencies, sodium currents suffer up to a 10-fold further reduction. Fig. 1 is from an axon containing 0.2 M internal sodium, and shows sodium currents as they diminish during successive 70-mV depolarizations given at 1-s intervals. Currents are inward (Fig. 1B) or outward (Fig. 1A and C) depending on whether or not sodium is present externally. Recovery at -70 mV from this accumulating block occurred with time constants $\simeq$ 20 s, regardless of external $[Na]^+$, and was too slow to be significant between pulses. Included are the first 5 (Fig. 1A), 8 (Fig. 1B), or 4 (Fig. 1C) sweeps in a train of 12 pulses, as well as the last sweep which indicates a steady state. The potential during the pulses was varied, but the first and last pulses were always 70 mV in amplitude. The larger the depolarization, the deeper became the block. Depth of block was expressed as the peak current remaining during the last pulse divided by that during the first. This ratio depends on the potential during intervening pulses in the form of sigmoid curves (Fig. 2B).

Effects of the kind shown in Fig. 1 and 2 have been investigated extensively, using QX-314

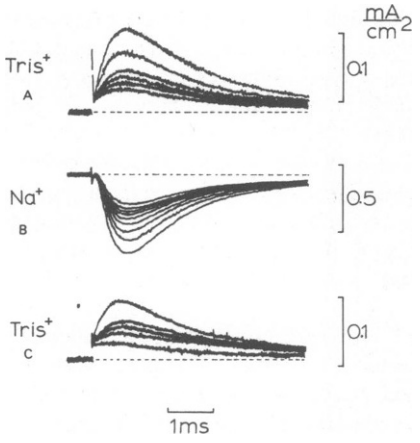


FIGURE 1

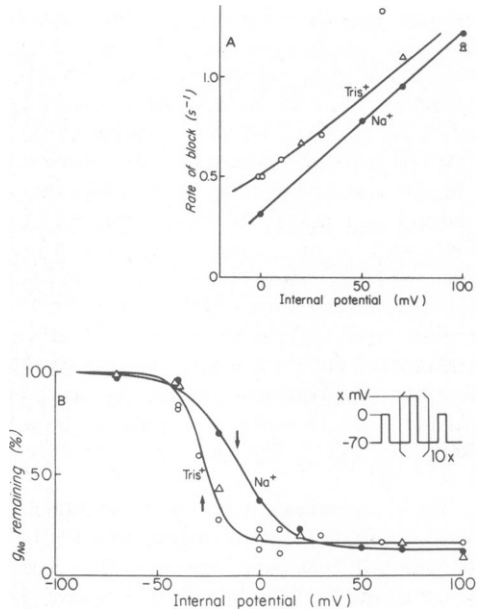


FIGURE 2

FIGURE 1 Effect of repetitive stimulation on sodium currents during 70-mV, 5-ms pulses to 0 mV. With each successive pulse, current is smaller. Internal solution contained 1 mM QX-314 as well as 0.2 m Na^+ (solution 200 Na^+). External solution: normal artificial sea water (Na-ASW) (B) or $Tris^+$-ASW (A and C); records were obtained in the sequence shown. Each panel shows only the first 5 (A), 8 (B), or 4 (C), as well as the last sweep in each train of 12 pulses. Axon 10; holding potential, −70 mV; 8°C.

FIGURE 2 Voltage and sodium dependence of block. Each point is from a train of 12 depolarizing pulses given at 1 Hz. The axon was allowed to rest for 2.5–3 min at −70 mV between trains. The first and last pulses went to O mV, the others to the potential on the abscissa (see inset). Circles represent measurements before, dots during, and triangles after presence of 0.44 M external sodium. (A) Currents at a fixed time after the start of each pulse were plotted against time after the first pulse in each train. Exponentials were fitted to these plots, and the ordinate gives the reciprocal time constants. Curves drawn by eye. (B) Peak current during the last pulse as a percentage of that during the first. Curves were drawn by Eq. 1 with $A = 0.84$, $B = 0.16$, $\overline{V} = -27.6$ mV, $s = 6.3$ mV (○, △) and $A = 0.87$, $B = 0.13$, $\overline{V} = -7.3$ mV, $s = 12.7$ mV (●). Arrows indicate $\overline{V}$. Same experiment as Fig. 1.

and other local anesthetics (Strichartz, 1973; Hille et al., 1975; Courtney, 1975; Khodorov et al., 1976; Hille, 1977; Schwarz et al., 1977; Cahalan, 1978). These analyses have shown that charged local anesthetics can leave or bind to a sodium channel only when it is open, and have led to the suggestion that the local anesthetic receptor lies inside the channel.

In Figs. 1 and 2, the main cation in the external solution was first the impermeant $Tris^+$ (open circles in Fig. 2), then sodium (filled circles) and then $Tris^+$ once more (open triangles). $Tris^+$ itself is inert (Begenisich and Cahalan, 1976)[1] as can be shown by comparing time-course and voltage dependence of amplitudes of sodium currents before and after replacing three-quarters of the external sodium with this organic cation. Fig. 2 shows that at a given potential, sodium diminishes both depth and rate of block. Evidently, external sodium

[1]Cahalan, M. D. Unpublished observations.

antagonizes internal QX-314 mainly by slowing the arrival rate of drug at the receptor. In potassium channels a similar effect was described by Armstrong (1971) with external potassium relieving K^+-channel block caused by internal tetraethylammonium ions (TEA^+). The effect was explained to result from K-ions knocking the blocking ion (TEA^+) off its receptor (Armstrong, 1971) or, more recently, by postulating multiple cation binding sites inside the channel which, for electrostatic reasons, cannot easily be occupied simultaneously (Hille and Schwarz, 1978). Similar explanations may apply here. The main conclusion, however, can be drawn from the simple fact that external sodium antagonizes block by internal QX-314. Because both ions are normally membrane-impermeant, the only plausible place where they can meet and interact is inside the sodium channel. The finding can be taken as evidence that the local anesthetic receptor lies there as well.

For a quantitative comparison, we assume that in the steady state, peak sodium conductance before ($g_{Na,rest}$) and after conditioning ($g_{Na,cond}$) are related to the potential V_c during conditioning pulses by:

$$\frac{g_{Na,cond}}{g_{Na,rest}} = \frac{A}{1 + \exp[(V_C - \overline{V})/s]} + B, \quad (1)$$

where $\overline{V}$ is the potential at the inflection point of the curves in Fig. 2B and s a parameter determining the steepness of these curves. Eq. 1 was previously used by Cahalan (1978) and, in a different form, by Strichartz (1973) to describe the voltage dependence of the drug-channel reaction. Such a voltage dependence would result if, before binding, a drug molecule had to penetrate some constant distance into the channel under the influence of the electric field. Then $\overline{V}$ gives the equilibrium potential for half-blockage and 25 mV/s is the fractional distance of penetration for a univalent blocking particle. The voltage dependence could also arise indirectly from voltage-dependent binding of a permeant cation to a site within the channel (Woodhull, 1973) if both cation and drug competed for the same site. Any voltage dependence derived from the opening and closing of channels is ignored in Eq. 1.

The curves in Fig. 2B are least-squares fits of Eq. 1 to the data; the computer was given independent choice of A, B, $\overline{V}$, and s. Table II summarizes our three experiments of this kind.

TABLE II
VOLTAGE AND SODIUM DEPENDENCE OF BLOCK

	Axon 5	Axon 9	Axon 10
$[Na]_i^+$, *mM*	50	200	200
Na^+ outside			
s, *mV*	32.5	23.3	12.7
$\overline{V}$, *mV*	−4.6	−2.9	−11.2
$Tris^+$ outside			
s, *mV*	6.27	6.27	6.33
$\overline{V}$, *mV*	−22.6	−30.6	−27.5

Axon 10 showed a voltage dependence steeper than expected from a univalent ion in simple models, and also steeper than observed previously by Strichartz (1973) and Cahalan (1978). This steepness could be an erroneous consequence of fitting the data with Eq. 1. Curves of voltage-dependent block were slightly asymmetrical, being relatively less steep at positive potentials where sodium channel gating may be thought to contribute less. If A and B are retained and the data are positive to −20 mV fitted by Eq. 1, s = 26 mV in axon 10, more similar to the results obtained in earlier studies.

Replacing external sodium with $Tris^+$ consistently shifts the voltage dependence to more negative potentials. The curve in $Tris^+$ is also steeper. Steepening could be a secondary consequence of shifting the occurrence of block into a potential range where the opening and closing of channels itself is strongly voltage dependent. If the drug binds only to open channels, one may expect that sodium channel gating will add its steep voltage dependence to that of the channel-drug reaction. Bezanilla and Armstrong (1977) have shown how even a voltage-independent reaction between a blocking particle and pore can derive a steep voltage dependence from the fact that the opening and closing of the unblocked pore is strongly voltage dependent.

The small conductance, *B*, remaining at the most positive potentials was not investigated systematically. It could represent partial recovery from block during the 1-s interval between pulses, or it could be due to a small population of channels that have lost their ability to inactivate for reasons not known with certainty. Such channels may be resistant against depolarization-induced block by QX-314 (Cahalan, 1978).

If block occurs only while the channel is open, rates of block must be much faster than the ordinate of Fig. 2 would suggest. Depolarizing pulses lasted 5 ms in these experiments, so sodium channels were open for < 5 ms out of every second. True entry rates for QX-314 are then expected to be at least 200 times faster than indicated in Fig. 2.

Block of Gating Current by QX-314

In further studies of depolarization-induced block by QX-314, we compared sodium currents and gating currents after a 1-min rest period ("resting" in Figs. 3 and 4) with those recorded

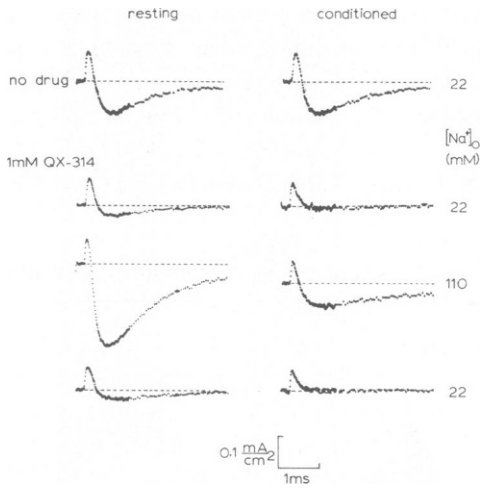


FIGURE 3 Stimulation—enhanced block of sodium—and gating currents by 1 mM QX-314. The figure shows signal-averaged currents during test pulses from −70 mV to +30 mV, either after a 1.5–2 min rest period (resting) or 50 ms after the last in a train of 10 depolarizing pulses from −70 to +95 mV ended (conditioned). Sodium and gating currents are reduced by the train of depolarizations. Each trace shows the average of one to five sweeps; resting and conditioned measurements alternated. For example, the traces in the last row were compiled from four resting and three conditioned current records, with intervals of 1.5–2 min between each. Axon 99; holding potential, −70 mV; Temp.; 8°C; *x* mM Na-ASW//200 Cs, where *x* is given next to each trace.

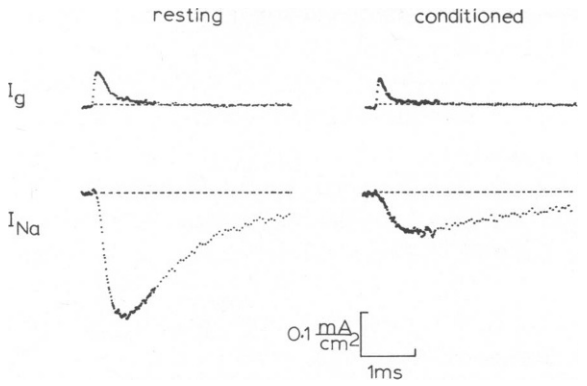
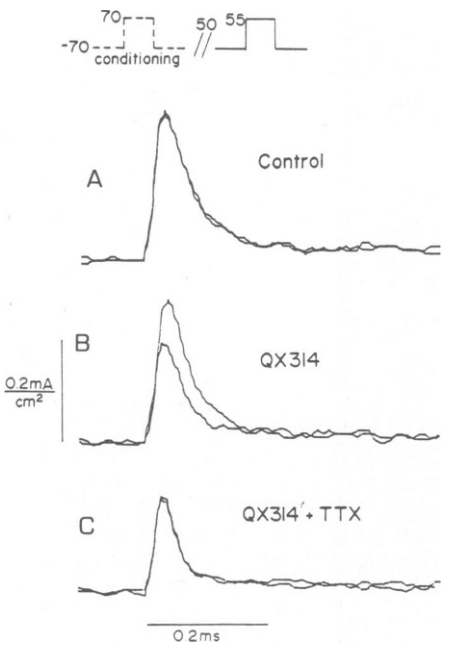


FIGURE 4 Separation of I_{Na} and I_g by the independence principle (Hodgkin and Huxley, 1952). In the absence of drug, the reversal potential for the sodium channel, V_{Na}, was 70 mV at $[Na]_0$ = 22 mM. From independence, I_{Na} at 110 mM $[Na]_0$ should then be 6.012 times larger than in 22 mM $[Na]_0$. Let I (22 mM) be the average of traces in the second and fourth row of Fig. 3, obtained by adding the two resting traces and dividing the result by two, and proceeding similarly with the conditioned traces. I (110 mM) are the traces in the third row of Fig. 3. Then the top traces in this figure (I_g) are formed by $I_g = 1.200 \times [I(22 \text{ mM}) - I(110 \text{ mM})/6.012]$, and the bottom traces by $I_{Na} = I(110 \text{ mM}) - I_g$.

50 ms after the end of a train of 10 conditioning pulses ("conditioned" in Figs. 3 and 4). Without drug the effects of such conditioning pulses reverse completely within 50 ms, whereas in the presence of QX-314, tens of seconds are required for recovery. Unfortunately, tetrodotoxin had to be avoided here for reasons given later, despite the difficulty of obtaining records of gating current uncontaminated by ionic currents. We describe two approaches (Figs. 3, 4, and 5); both gave qualitatively the same result.

Fig. 3 shows gating currents and sodium currents during pulses from −70 to 30 mV. The experiment starts with $[Na]_0$ = 22 mM; outward gating current is nearly as large as the subsequent sodium inward current. In the absence of drug, conditioning is without effect. After 1 mM QX-314 is added internally, sodium current is diminished (left) and becomes almost totally blocked by conditioning pulses (right). Measurements were repeated at higher $[Na]_0$ (110 mM) and then once more at $[Na]_0$ = 22 mM. Conditioning pulses went to 90 mV, where block by QX-314 is no longer strongly dependent on $[Na]_0$ (Fig. 2). The total current may be separated into sodium current and gating current components if (*a*) gating current is independent of $[Na]_0$ over the range explored here and (*b*) the "independence principle" of Hodgkin and Huxley (1952*a*) holds. For this experiment, the independence principle predicts that raising $[Na]_0$ 5-fold should increase sodium inward current 6.0-fold at +30 mV. The two sets of traces at 22 mM $[Na]_0$ were averaged, and the middle traces (110 mM) were divided by 6.0 and then subtracted from the set at 22 mM $[Na]_0$. The results—traces of "pure" gating current (I_g)—were scaled to restore the original gain, and are shown in Fig. 4. Subtracting them from the 110-mM traces in Fig. 3 yields pure sodium current (I_{Na}) at 110 mM $[Na_0]$, also shown in Fig. 4. In this experiment, conditioning reduced peak sodium current 3.3-fold, and gating charge movement was reduced from 22.1 to 13.8 nC/cm^2. Among the sources of error in this experiment are: (*a*) inaccuracies in the measurement of the sodium reversal potential V_{Na}, (*b*) deviations from the independence principle (e.g., Fig. 5 of Hille, 1975, for data over the present concentration range), and (*c*) fiber rundown during the experiment.



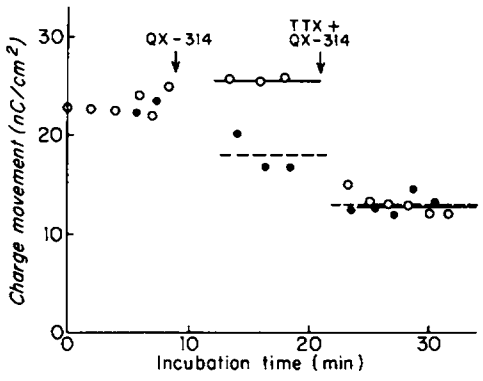


FIGURE 5

FIGURE 6

FIGURE 5 Membrane currents during depolarization to 55 mV. Because 55 mV was the approximate value of V_{Na} in this fiber, sodium current will not contribute significantly, and the outward transients should be predominantly gating currents. Resting and conditioned traces are superimposed. Each trace was compiled, by signal-averaging, from two to five sweeps stored individually on DEC-tape; resting sweeps were preceded by a 1-min rest period, conditioned sweeps by a train of 21 pulses to +70 mV and then a recovery interval of 50 ms. Resting and conditioned measurements alternated, as in Fig. 3. Control, no drug; QX-314, 1 mM internal QX-314; QX-314 + tetrodotoxin (TTX), 1 mM internal QX-314 + 1 μM external TTX. 22 mM Na-ASW//200 Cs. Axon 203; holding potential, −70 mV; 16°C.

FIGURE 6 Analysis of Fig. 5. Charge movement obtained by numerical integration of outward current transients. ○, resting; •, conditioned measurements. Horizontal lines indicate averages for each condition. Drugs were added at the times indicated: 1 mM QX-314 internally and 1 μM TTX externally. Measurements in the absence of drug (leftmost eight points) seem lower than resting measurements in QX-314; this may be due to removal, by QX-314, of a small contaminating sodium current.

However, all these errors are too small, and error (*b*) in the wrong direction, to endanger the main conclusion: when block by QX-314 is induced by conditioning pulses, part of the gating charge movement is blocked as well.

Fig. 5 illustrates a different kind of experiment leading to the same conclusion. Gating currents are recorded during steps from −70 mV to the approximate V_{Na} in this fiber, namely 55 mV. Temperature was raised to increase the relative size of gating current. In the absence of drug (Fig. 5A), conditioned and resting traces superimpose. Adding 1 mM QX-314 has little effect at rest, but after conditioning, gating currents are smaller, confirming the experiment in Fig. 4.

Interaction between Tetrodotoxin and QX-314

Fig. 5C also shows a new effect of tetrodotoxin. After the toxin is added externally (0.1 μM) in addition to the internal QX-314, gating current appears reduced even at rest, and

TABLE III

EFFECT OF CONDITIONING, QX-314 AND TETRODOTOXIN ON CHARGE MOVEMENT

Axon	99	100	103	104	203	204	Mean ± SEM
No drug							
I_{Na} ratio	0.96	0.95	—	1.02	—	—	0.98 ± 0.02
Q_{rest}, nC/cm^2	—	13.4	14.1	12.3	23.8	28.9	—
Q ratio	—	—	—	1.03	0.96	1.02	1.00 ± 0.02
1 mM QX-314							
I_{Na} ratio	0.30	0.27	—	0.18	0.25	—	
Q_{rest}, nC/cm^2	22.1	19.4	—	12.4	25.7	25.9	
Q ratio	0.62	0.49	—	0.64	0.70	0.69	0.63 ± 0.04
1 mM QX-314 + 1 μM TTX							
Q_{rest} nC/cm^2	8.7	7.9	7.2	5.4	12.8	15.0	
Q ratio	0.92	0.90	0.97	0.96	1.01	0.92	0.94 ± 0.02
Temp, °C	8	8	8	8	16	16	
V_{Na}, mV		70	60	68	55	55	

For details of axon 99 see legend to Figs. 3 and 4. Experiments of axons 100–204 similar to Fig. 5; gating currents were measured during pulses to approximately the sodium reversal potential V_{Na} (last row). Peak sodium currents were measured during pulses to 10–30 mV; their ratio (conditioned:rest) is given (I_{Na} ratio). Charge movement (Q) was measured by numerical integration over the first 0.5 ms (axons 203, 204) or 1-1.3 ms (axons 99–104) after beginning of the pulse; a horizontal base line fitted to the 1–2 ms after the integration interval was subtracted before integration took place. Q_{rest}, charge movement after 1 min rest at −70 mV; Q ratio, Q_{cond}:Q_{rest}, where Q_{cond} was obtained after a train of conditioning pulses to 70–100 mV. After conditioning in the presence of QX-314 alone, a 3–5-fold reduction of I_{Na} is accompanied by 1.4–2-fold reduction in charge movement. This effect is always absent when TTX is present also. Instead, charge movement is 0.46 ± 0.03 times the resting charge movement measured when only one or no drug is present (± SEM, n = 5, axons 99–204). 22 mM Na^+ − ASW//200 Cs; holding potential, −70 mV; temperature indicated.

conditioning causes no further reduction. The system behaves as if QX-314 is now permanently locked into the channel. Fig. 6 retraces the history of this experiment and compares charge movement in the resting (O) and conditioned (●) fiber. In the absence of drug, charge movement is large and independent of conditioning; adding QX-314 causes block after a train of depolarizations, and when toxin and drug are present simultaneously charge is always small, regardless of conditioning. Tetrodotoxin alone does not measurably reduce charge movement (one experiment at 8°C, pulses to V_{Na} at 22 mM $[Na]_o$; see also Armstrong and Bezanilla, 1974). Table III summarizes other experiments supporting the findings of Fig. 5. It seems as though external tetrodotoxin makes it easier for QX-314 to remain in the channel.

In one experiment, we recorded gating currents over a large range of potentials, first with external tetrodotoxin, and then in addition with internal QX-314 (Fig. 7). As in Figs. 4 and 10, QX-314 diminishes the amplitude of gating currents as well as speeding their rise and decline. The charge-voltage curve for this experiment (Fig. 8) was shifted to more positive potentials by the combination of drugs.

Physiological and Drug-Induced Charge Immobilization

Recovery from charge immobilization was studied with two-pulse experiments as in Fig. 9. The first pulse was a 5-ms depolarization to 70 mV causing maximal sodium current inactivation and charge immobilization; the second pulse went to 30 mV and started after the

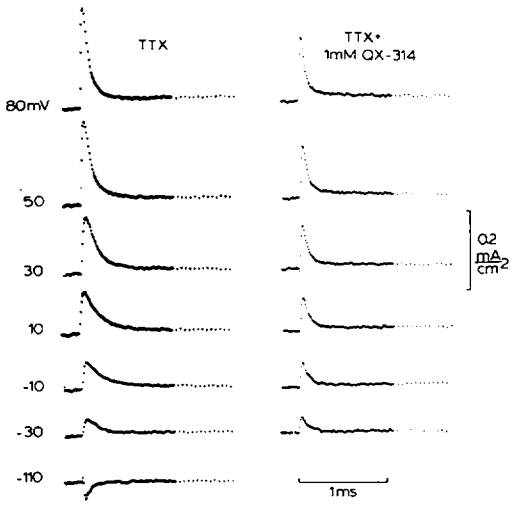


FIGURE 7

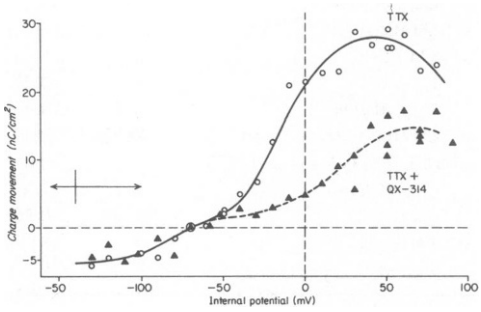


FIGURE 8

FIGURE 7 Gating currents in the presence of external TTX (1 μM) and, in addition, internal QX-314 (1 mM). Pulses went from −70 mV to the potentials indicated. Each trace signal-averaged five times; pulses were given at 0.1-s intervals during averaging. Control pulses started from −140 mV in this experiment. Axon 109; holding potential, −70 mV; 22 mM Na-ASW//200 Cs; 8°C.

FIGURE 8 Charge-voltage curves with and without QX-314. Charge movement measured by numerical integration over the first 1.2 ms after depolarization. Before integration, sloping base lines fitted to the 0.6 ms following the integration interval were subtracted from each record. Points on each curve were obtained in the sequence −30, −10, +10 mV etc., and then −40, −20, 0, +20 mV etc. Curves drawn by eye. Arrow at left indicates the voltage range spanned by control pulses. We have no satisfactory explanation for the apparent decline of charge movement at the most positive potentials. It may be a consequence of gating current during the control pulse (Adrian and Almers, 1976). Same experiment as in Fig. 7.

interval indicated on the abscissa. During the interval, peak sodium current (Fig. 9A) and charge movement (Fig. 9B) recover in parallel. The curves without QX-314 are least-squares fits of single exponentials; their time constants are τ_h = 3.8 ms (Fig. 9A) and τ_Q = 3.6 ms (Fig. 9B). In 5 experiments of this kind, the ratio of time constants τ_h:τ_Q was 1.32 ± 0.10 SEM at 8°C and −70 mV. After 1-ms recovery, sodium current was 0.24 ± 0.04 and charge movement 0.40 ± 0.03 of the maximum in these experiments. Comparing fractional inactivation and charge immobilization after 1-ms recovery suggests that for complete inactivation, charge immobilization would have been 79 ± 8% complete. These data agree well with the earlier and more extensive measurements of Armstrong and Bezanilla (1977), whose data (their Table V) indicate τ_h:τ_Q = 1.26 ± 0.17 (n = 5) at 8°C and −70 mV. The deviation of τ_h:τ_Q from unity in our experiments may be statistically significant, but it is small and could represent an effect of tetrodotoxin on the inactivation process, or else be due to progressive changes in the axon, since τ_h was generally measured first.

Fig. 9 also compares charge movements with and without QX-314 (Fig. 9B). Tetrodotoxin was present throughout. As before, maximal charge movement (dotted lines) with drug and toxin is less than with toxin alone. The effect of a prepulse in causing immobilization is also less. The two exponentials (both with time constant τ_Q = 3.6 ms) extrapolate to similar values

at $t = 0$, suggesting that physiological and drug-induced charge immobilization are not additive. A similar experiment at higher temperature is shown in Figs. 10 and 11. Fig. 10 shows asymmetry currents with (B) and without (A) an inactivating prepulse, first without any drugs and then with both QX-314 and tetrodotoxin. As before, the pulse went to +55 mV, roughly equal to V_{Na} in this fiber. Once more, the charge movement remaining after adding the two drugs is resistant to immobilization and seems in size and time-course identical to what remains, in the absence of drugs, after an inactivating prepulse. Fig. 11 analyzes the experiment; without drug, immobilized charge recovers, whereas in presence of tetrodotoxin and QX-314 it does not. In other experiments with QX-314 alone, we found that it makes little difference to the charge movement whether the recovery interval from a preceding train of depolarizations is 1 or 50 ms. Our data therefore suggest that the component of charge movement immobilized by QX-314 is the same as the component subject to physiological immobilization.

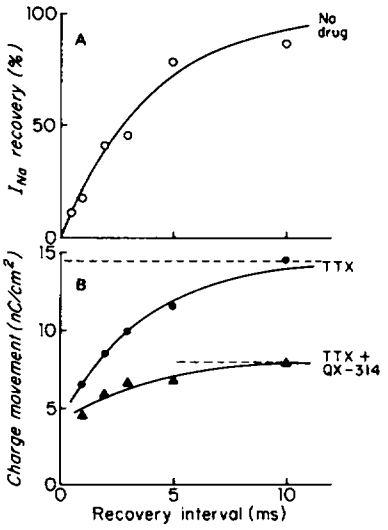


FIGURE 9

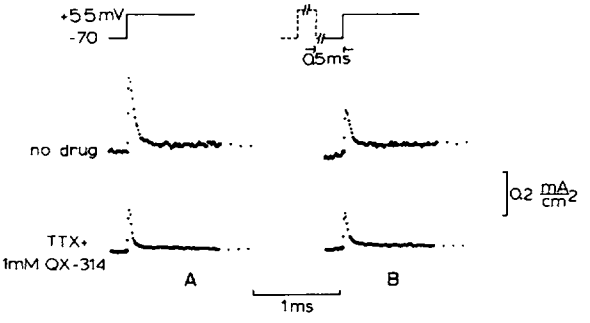


FIGURE 10

FIGURE 9 Recovery from inactivation (A) and charge immobilization (B) by a 5-ms prepulse to 70 mV. The ordinates give peak sodium current (A) and charge movement (B) during a second pulse, this time to +30 mV, which followed the end of the first after the interval indicated on the abscissa. We measured sodium currents in drug-free solution (110 mM Na-ASW//200 Cs), then gating currents in 22 mM Na-ASW//200 Cs and 0.5 μM external TTX, then gating currents in 22 mM Na-ASW//200 Cs after adding 1 mM internal QX-314. Gating currents were signal-averaged 5 (TTX) or 10 times (TTX + QX-314) with 0.1-s intervals between each sweep. Charge movements without prepulse were 14.6 nC/cm^2 (TTX) or 8.0 nC/cm^2 (TTX + QX-314). Curves without QX-314 are least-squares fits and given by [1-exp($-t$/3.8 ms)] (no drug) and by {10.7[1-exp($-t$/3.6 ms)] + 3.9} nC/cm^2 (TTX). The curve through triangles results from assuming the time constant of recovery to be unchanged by the drug, and is given by {4.1[1-exp($-t$/3.6 ms)] + 3.9} nC/cm^2. This assumption would be correct if the charge movement recovering here (triangles) is due to channels that remain unblocked at 1 mM QX-314. Axon 107; holding potential, −70 mV; 8°C.

FIGURE 10 Gating currents during a pulse to 55 mV without (A) or with (B) an inactivating 5-ms prepulse to 70 mV. Interval between pulses, 0.5 ms, is indicated above the traces. Top traces: drug-free media, no signal-averaging. Bottom traces: 1μM TTX plus 1 mM QX-314, signal-averaged five times as in Fig. 9. Same experiment as in Fig. 5 (16°C).

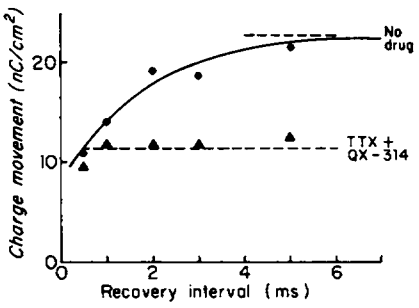


FIGURE 11

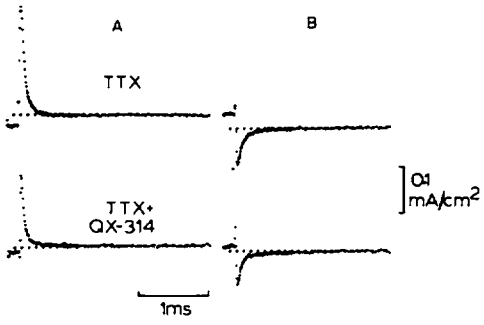


FIGURE 12

FIGURE 11 Recovery of charge movement from immobilization by a 5-ms prepulse to 70 mV. Dashed lines indicate charge movements without prepulses. The single exponential drawn through the dots is a least-squares fit; its time constant is 1.73 ms. Measurements with TTX + QX-314 were signal-averaged (Fig. 9); those without drug were not. For further details see legend of Fig. 5.

FIGURE 12 Gating currents during (A) and after (B) a 5-ms pulse from −70 to +70 mV. Control pulses from −140 to −105 mV. In each of the four traces, sloping base lines were fitted to the section between 1.6 and 3.1 ms after the pulse started. These were subtracted before display. The on transients carried 23.4 (top) and 16.6 nC/cm² (bottom), the off transients 10.8 (top) and 11.7 nC/cm² (bottom). In this experiment QX-314 evidently failed to cause complete block of the immobilizable component. The off figures include a slow component of charge movement of unknown significance often seen by us. Same experiment as in Figs. 7 and 8 where details are given.

Further support for this conclusion comes from Fig. 12, which shows gating current during ("on," A) and after ("off," B) a depolarizing pulse of sufficient duration to cause pronounced charge immobilization. Adding internal QX-314 strongly diminishes gating current during the pulse, but there is less effect on that after the pulse. Once again, the drug attacks the immobilizable, but not the immobilization-resistant component of charge movements. As far as gating currents are concerned, a channel blocked by QX-314 evidently behaves like an inactivated channel.

The preferential block of the on gating current in Fig. 12 stands in marked contrast to the effects of pancuronium (Armstrong and Yeh, 1978) and *N*-methylstrychnine (Cahalan and Almers, 1979). These two drugs block "off" gating current almost completely, with little or no effect on the on gating current. This difference will be discussed in the following paper and probably arises because QX-314-occupied channels can close readily, whereas channels blocked by pancuronium (Yeh and Narahashi, 1977) or *N*-methylstrychnine (Cahalan and Almers, 1979) cannot.

DISCUSSION

The Receptor for Quaternary Lidocaine

This paper investigates depolarization-induced block of sodium channels by QX-314, a permanently charged, membrane-impermeant (Frazier et al., 1970) derivative of the local anesthetic, lidocaine. Previous authors have suggested that the voltage-dependent effects of quaternary lidocaine (Strichartz, 1973) or local anesthetics in general (Courtney, 1975; Hille, In Strichartz's (1973) model, the receptor can be reached only when the channel gates are

open, and the QX-314 molecule is viewed to block ion flow simply by obstructing the aqueous pore. This model closely resembles one developed by Armstrong (1971) for the effect of quaternary ammonium compounds on K^+ channels, in that obstruction of the pore, the electric field and interaction with the gating mechanism combine in producing voltage- and time-dependent block. Later models (Courtney, 1975; Hille, 1977) specify the interaction with channel gates in greater detail and postulate that local anesthetics block by promoting inactivation. In Hille's model (1977) the binding site inside the aqueous pore is retained, but the concept of block by obstruction is no longer essential (an inactivated channel will not pass current, regardless of whether or not there is an additional obstruction of the aqueous pore by a local-anesthetic molecule). The idea that some local anesthetics can block sodium channels by obstruction nevertheless remains attractive, since some drugs alter the activation kinetics in a characteristic manner (Courtney, 1975) and others continue to give voltage- and time-dependent block (Cahalan, 1978) when inactivation is prevented by mild internal digestion with pronase (Armstrong et al., 1973). For QX-314, block by obstruction has not been established with certainty.

Our gating current measurements confirm that QX-314 interacts with the channel gates, and the antagonism between internal QX-314 and external sodium moves us to accept, at least as a working hypothesis, the presence of a receptor inside the aqueous pore. The alternative explanation for the antagonism, namely an allosteric effect of external sodium on an internal QX-314 receptor, seems to us unnecessarily complicated and, in its vagueness, less useful in designing further experiments. For the remainder of this discussion, we will thus attribute the voltage- and time-dependent effects of the drug to a "local anesthetic receptor" inside the aqueous pore, bearing in mind the possibility that future experiments may make it necessary to postulate further receptors elsewhere.

Partial Block of Gating Current by Local Anesthetics

Inactivation and, as we show here, the voltage-dependent block by QX-314 are both accompanied by immobilization of gating charge. Thus a connection between inactivation and partial block of gating current by QX-314 seems likely and remains so even upon closer investigation. Both QX-314 and inactivation appear to act on the same fraction of charge movement inasmuch as their effects are not additive. Moreover, charge movements remaining after block by QX-314 and inactivation are closely similar in time-course (c.f., Fig. 10). After block by QX-314, charge movements show an accelerated rising phase (Figs. 7 and 10) and a more rapid rate of decline; both features are also seen after inactivation (Fig. 5 of Armstrong and Bezanilla, 1977 and, in myelinated nerve, Fig. 7 of Nonner, 1978). Judging from gating currents, QX-314 either promotes or simulates inactivation.

Charge immobilization by QX-314 is consistent with previous work. Keynes and Rojas (1974) have observed that 40 mM external procaine speeds gating charge movement and reduces it threefold. Peganov and Khodorov (1977) have recently reported that trimecaine also reduces gating charge movement, and have suggested that gating currents through trimecaine-blocked and normal but inactivated sodium channels are similar. The work in both of these papers was carried out in the presence of tetrodotoxin. By initially avoiding the toxin, we could show that, as with sodium currents, block of gating currents by QX-314 depends on conditioning depolarizations and that tetrodotoxin and QX-314 can act synergistically in

causing block. There remains thus little doubt that the effects of QX-314 on sodium and gating currents are linked. The results on the three drugs procaine, trimecaine, and QX-314 are in good agreement, and it seems possible that their effects on gating currents have a common molecular basis.

In addition to the voltage- and time-dependent block produced by repetitive depolarization, QX-314 and other local anesthetics also cause a "tonic" component of block that appears even in a resting axon. In myelinated nerve, at least part of the block induced by tertiary amine local anesthetics in resting axons can be reversed by hyperpolarization (Khodorov et al., 1976; Hille, 1977), and this portion may arise in the same way as the depolarization-induced block (Hille, 1977). In many of our experiments (e.g., Fig. 3), QX-314 caused an up to twofold tonic reduction of sodium current, which, unlike the depolarization-enhanced block, is not accompanied by measurable charge immobilization (compare data for resting axons in Figs. 5 and 6) even though in absolute terms, tonic and voltage-dependent conductance deficits were of similar magnitude. This suggests different mechanisms (and receptors?) for tonic- and voltage-dependent block. Both kinds of block are also affected differently by pronase treatment; voltage-dependent block by QX-314 and QX-222 is abolished, but a large tonic reduction of sodium current remains at physiological potentials (Fig. 6 in Cahalan, 1978). A second receptor responsible for tonic block need not be part of the sodium channel. For instance, nonspecific adsorption of local anesthetic to the lipid bilayer could diminish the conductance of an open sodium channel, thus reducing the macroscopic conductance (Almers, 1976).

Comparing drug-induced and physiological charge immobilization in the light of Armstrong and Bezanilla's (1977) hypothesis, one may suggest that either (*a*) the QX-314 molecule substitutes for the physiological blocking (or inactivating) particle, or (*b*) drug-induced immobilization is caused by the physiological blocking particle, and the main action of the drug molecule inside the channel is to stabilize the association between channel and inactivating particle (Courtney, 1975; Hille, 1977). Hypothesis *b* seems preferable. If *a* applied, one might expect that QX-314 and physiological blocking particle should compete with one another, as is the case with pancuronium (Yeh and Narahashi, 1977) and *N*-methylstrychnine (Cahalan and Almers, 1979). For instance, pronase treatment might be expected to free QX-314 from competition with the physiological blocking particle and should make QX-314 more, rather than less, effective. Instead, pronase-treatment protects against depolarization-induced block by QX-314, as if an intact inactivation mechanism is required for QX-314 action (Cahalan, 1978). This argument is not conclusive, however, because pronase treatment may remove or modify the local-anesthetic receptor.

Pursuing hypothesis *b*, it is interesting that the presence of QX-314 and physiological blocking particle inside the channel appears to cause no more charge immobilization than the blocking particle alone. One possible explanation is that under both conditions immobilization of the gating machinery is complete, and that the immobilization-resistant charge movement is unrelated to sodium channels (Peganov and Khodorov, 1977). However, pancuronium (Armstrong and Yeh, 1978) and *N*-methylstrychnine (Cahalan and Almers, 1978) can block also the normally immobilization-resistant fraction of gating charge movement as they enter sodium channels. These findings suggest that at least part of the immobilization-resistant charge component is related to sodium channels.

Interaction with Tetrodotoxin

Past experiments have shown a remarkable lack of interaction between the tetrodotoxin receptor and the rest of the sodium channel. Toxin binding in itself does not strongly alter gating currents (Armstrong and Bezanilla, 1974), and membrane depolarization sufficient to open and inactivate normal sodium channels does not measurably affect toxin binding (Almers and Levinson, 1975). Here we show for the first time that besides blocking ionic current, the toxin can influence the action of intracellular drugs and, indirectly, the gating of pharmacologically altered channels. Our finding may help to explain why pharmacological effects on sodium current kinetics do not always correlate quantitatively with effects on gating currents observed in the presence of tetrodotoxin. Possible examples besides local anesthetics are pancuronium (Armstrong and Yeh, 1978) and perhaps also dichloro-diphenyl-trichloroethane (DDT) (Dubois and Bergman, 1977).

Tetrodotoxin's enhancement of block by QX-314 seems surprising, because both substances bear a positive charge and ought to repel one another. If the toxin slowed the dissociation rate without speeding the arrival rate, the slowing must have been at least approximately fivefold, otherwise measurable recovery should have occurred during the 1–2 min rest periods. It would be of interest whether the synergism between QX-314 and tetrodotoxin is mutual. Lidocaine (1 mM) reportedly does not change the binding of tritium-labeled tetrodotoxin to the rabbit vagus nerve (Colquhoun et al., 1972), but the point could be reinvestigated with improved methods (Ritchie et al., 1976).

The synergism between toxin and drug might be explained if tetrodotoxin binding to the channel caused a conformational change that allosterically encouraged QX-314 to bind. Alternatively, it may be related to the antagonism between QX-314 and external sodium. The antagonism suggests the presence of two or more cation binding sites in the channel that cannot easily be occupied simultaneously. It seems possible that dissociation of QX-314 normally results from electrostatic repulsion by a sodium ion bound to a neighboring site. Binding of tetrodotoxin will cut off the external arrival route to this site for sodium or other permeant cations. When a QX-314 molecule has bound to (and blocked) a vacant and toxin-bearing channel, the only remaining internal access route is also obstructed, the vacancy cannot be filled, and dissociation of the drug must rely on thermal agitation alone. In any case, the stability of QX-314 binding in the presence of tetrodotoxin seems worthy of further attention. The drug-toxin combination may be useful to biochemists as a means for chemically locking the channel into a state resembling physiological inactivation.

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