Alkaloid-modified Sodium Channels from Lobster Walking Leg Nerves in Planar Lipid Bilayers

CECILIA CASTILLO, RAIMUNDO VILLEGAS, and ESPERANZA RECIO-PINTO

From the Departments of Anesthesiology and Physiology, Cornell University Medical College, New York 10021; and Instituto Internacional de Estudios Avanzados (IDEA), Caracas 1015A, Venezuela

ABSTRACT Alkaloid-modified, voltage-dependent sodium channels from lobster walking leg nerves were studied in planar neutral lipid bilayers. In symmetrical 0.5 M NaCl the single channel conductance of veratridine (VTD) (10 pS) was less than that of batrachotoxin (BTX) (16 pS) modified channels. At positive potentials, VTD- but not BTX-modified channels remained open at a flickery substate. VTD-modified channels underwent closures on the order of milliseconds (fast process), seconds (slow process), and minutes. The channel fractional open time (f_o) due to the fast process, the slow process, and all channel closures (overall f_o) increased with depolarization. The fast process had a midpoint potential (V_a) of -122 mV and an apparent gating charge (z_a) of 2.9, and the slow process had a V_a of -95 mV and a z_a of 1.6. The overall f_o was predominantly determined by closures on the order of minutes, and had a V_a of about -24 mV and a shallow voltage dependence ($z_a \approx 0.7$). Augmenting the VTD concentration increased the overall f_o without changing the number of detectable channels. However, the occurrence of closures on the order of minutes persisted even at super-saturating concentrations of VTD. The occurrence of these long closures was nonrandom and the level of nonrandomness was usually unaffected by the number of channels, suggesting that channel behavior was nonindependent. BTX-modified channels also underwent closures on the order of milliseconds, seconds, and minutes. Their characterization, however, was complicated by the apparent low BTX binding affinity and by an apparent high binding reversibility (channel disappearance) of BTX to these channels. VTD- but not BTX-modified channels inactivated slowly at high positive potentials (greater than $+30$ mV). Single channel conductance versus NaCl concentrations saturated at high NaCl concentrations and was non-Langmuirian at low NaCl concentrations. At all NaCl concentrations the conductance of VTD-modified channels was lower than that of BTX-modified channels. However, this difference in conductance decreased as NaCl concentrations neared zero, approaching the same limiting value. The permeability ratio of sodium over potassium obtained under mixed ionic conditions was similar for VTD (2.46)- and BTX (2.48)-modified channels, whereas that obtained under bi-ionic conditions was lower for VTD (1.83)- than for BTX

Address reprint requests to Dr. Esperanza Recio-Pinto, Department of Anesthesiology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

(2.70)-modified channels. Tetrodotoxin blocked these alkaloid-modified channels with an apparent binding affinity in the nanomolar range.

INTRODUCTION

Voltage-dependent sodium channels show functional variability both between and within tissues (Gilly and Armstrong, 1984; Sherman and Catterall, 1985; Kirsch and Brown, 1989; Patlak and Ortiz, 1989). This variability may provide different excitable cells and cell regions with specificity for carrying out their distinct cellular functions, as well as their responsiveness to endogenous and exogenous factors. Sodium channel function depends on channel structure, including amino acid sequence (Stühmer, Conti, Suzuki, Wang, Noda, Yahagi, Kudo, and Numa, 1989), sugar composition (Levinson, Thornhill, Duch, Recio-Pinto, and Urban, 1990; Recio-Pinto, Thornhill, Duch, Levinson, and Urban, 1990), and channel lipid environment (Feller, Talvenheimo, and Catterall, 1985; Duch and Levinson, 1987b). Therefore, to study the function of a specific channel structure it is essential to control the channel's lipid environment. This can be done with the planar lipid bilayer system.

The alkaloid binding site (as yet unidentified channel structure) is of great interest because it is capable of modulating various aspects of sodium channel function. In stimulating Na^{22} flux in vesicles containing sodium channels from lobster leg nerves, it has been shown that, compared with vesicles containing channels from other sources (Rosenberg, Tomiko, and Agnew, 1984; Tamkun, Talvenheimo, and Catterall, 1984; Tanaka, Furman, and Barchi, 1986; Duch and Levinson, 1987a), the alkaloid batrachotoxin (BTX) has a lower potency (Villegas and Villegas, 1981) and the alkaloid veratridine (VTD) has a similar potency (Correa, Villegas, and Villegas, 1987). Therefore, the functional properties of single sodium channels from lobster leg nerves were characterized in neutral lipid planar bilayers to investigate the underlying mechanisms responsible for the observed differences during flux studies, and to provide more information on the function of these channels.

Previous studies have shown that in neutral lipid planar bilayers various sodium channels are modified by micromolar concentrations of VTD and almost irreversibly by nanomolar concentrations of BTX (Moczydlowski, Garber, and Miller, 1984; Hartshorne, Keller, Talvenheimo, Catterall, and Montal, 1985; French, Krueger, and Worley, 1986a; French, Worley, Blaustein, Romine, Tam, and Krueger, 1986b; Garber and Miller, 1987; Green, Weiss, and Andersen, 1987a; Recio-Pinto, Duch, Levinson, and Urban, 1987; Duch, Recio-Pinto, Frenkel, and Urban, 1988; Behrens, Oberhauser, Bezanilla, and Latorre, 1989; Corbett and Krueger, 1989; Duch, Recio-Pinto, Frenkel, Levinson, and Urban, 1989). In voltage-clamped biological membranes, a fast developing alkaloid-modified current has been detected with VTD and BTX, and a slowly developing one with VTD (Ulbricht, 1972; Khodorov and Revenko, 1979; Huang, Moran, and Ehrenstein, 1982; Dubois, Schneider, and Khodorov, 1983; Leibowitz, Sutro, and Hille, 1986; Sutro, 1986; Barnes and Hille, 1988; Rando, 1989). The voltage dependence of the former process resembles that reported for the activation process of BTX-modified channels, while that of the latter process resembles the one reported for the overall fractional open time of VTD-modified channels in planar bilayers.

We now report that VTD-modified channels from lobster leg nerves undergo

closures on the order of milliseconds, seconds, and minutes, and inactivate slowly at high positive potentials (greater than +30 mV). Channel fractional open time due to closures on the order of milliseconds or seconds had a relatively sharp voltage dependence, which increased with depolarization. When modified with BTX, channels from lobster leg nerves underwent similar types of closures, but did not inactivate. The characterization of these closures was complicated by an apparent high binding reversibility (channel disappearance) of BTX to these channels. Our data, in conjunction with that of previous studies, indicate that based on alkaloid responsiveness there are at least two major types of sodium channels, one type which binds BTX essentially irreversibly and with high affinity, and another type which binds BTX with an apparent high reversibility and low affinity. Both types of channels appear to bind VTD with similar affinities.

METHODS

Preparation of Sodium Channel Material

Nerves were dissected from lobster walking legs of the species *Panulirus argus*, and their plasma membranes were isolated as previously described (Villegas, Sorais-Landaez, Rodriguez-Grille, and Villegas, 1988). The isolated plasma membranes were resuspended (3–4 mg protein/ml) in sucrose buffer (0.33 M sucrose, 25 mM potassium phosphate, pH 7.4) and stored at −80°C. To minimize degradation of sodium channel proteins, all the steps in the preparation of plasma membranes were carried out at 2°C, and in the presence of a mixture of protease inhibitors (5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 1 μM pepstatin A, 1 mM iodoacetamide, 1 μg/ml antipain, 1 μg/ml phosphoramidon, 20 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, 100 μg/ml bacitracin). There were no detectable differences in the alkaloid-modified sodium channel functionality of the four membrane preparations used in this study.

Channel Incorporation into Planar Lipid Bilayers

Planar lipid bilayers were formed as previously described (Recio-Pinto et al., 1987). Planar bilayers were formed across a hole (~0.2–0.3 nm in diameter) in a teflon partition. Neutral phospholipid solutions, used for forming the lipid bilayers, have phosphatidylcholine/phosphatidylethanolamine (1:4) in decane (5% wt/vol). The compartments on either side of the teflon partition (1 ml capacity) contained electrolyte solutions and sodium channel activating alkaloids. BTX (1–5 μM) was added only to the *trans* compartment, while VTD (500 μM unless otherwise indicated) was added to both compartments. When high BTX concentrations were used (25 μM), the required volume of the ethanolic BTX solution was placed in a plastic conical tube and evaporated to a volume of ~3 μl before its addition to the bilayer compartment. Channels were incorporated by adding the suspension of membrane fragments (~0.5–2.0 μl) directly above the partition hole alone, or mixed (1:1) with the phospholipid solution (Recio-Pinto et al., 1987), giving a final protein concentration of 2–8 μg/ml. In most experiments, high concentrations of tetrodotoxin (TTX; 30–150 μM) were added to one of the chamber compartments to ensure the study of channels incorporated into the bilayer in only one direction. Experiments were conducted at room temperature (22–25°C). Solutions were buffered to pH 7.4 with 10 mM HEPES.

A two-electrode voltage clamp was used (Dagan 3900, Dagan Corp., Minneapolis, MN; or EPC7 patch clamp, List Electronics, Darmstadt, Germany) with a current-to-voltage amplifier with a 10-GΩ resistor in the feedback loop. Ag/AgCl electrodes made direct contact with both aqueous solutions. The output from the voltage clamp was split between a DAS/VCR 900 digital

recorder (unfiltered or filtered 2–5 kHz) and an 8-pole Bessel filter (50–100 Hz). The output from the 8-pole Bessel filter was split between a microcomputer data acquisition (12-bit) interface (IBX box, Indec systems, Inc., Sunnyvale, CA; or Axolab, Axon Instruments, Inc., Foster City, CA) and a strip chart recorder (100–150-Hz response). The data acquisition and analysis were done using the PCLAMP programs (Axon Instruments, Inc.) and IBM-AT microcomputers. Data collected exclusively on strip charts were analyzed manually.

Reversal potential measurements under mixed ionic conditions were done in the following manner. Channels were first incorporated into membranes in symmetrical 0.5 M NaCl and their slope conductance and orientation were determined. Afterward, the concentration of NaCl was increased to 810 mM in the chamber where the extracellular aspect of the channel was facing, and KCl was added to the other chamber to give a final electrolyte composition of 450 mM NaCl and 360 mM KCl. When reversal potential measurements were made under bi-ionic conditions, the contribution of the junction potential was eliminated by zeroing the Ag-AgCl electrodes in symmetrical solutions of NaCl before replacing the solution in the *trans* chamber with KCl as previously described (Duch et al., 1989).

The relationship of channel conductance versus NaCl concentrations was studied with electrolyte solutions containing the indicated NaCl concentration without a supporting electrolyte; that is, at a varying ionic strength. The data were fitted to Eq. 4 using a nonlinear least-squares fit, and the line was drawn by providing the computer with a $[Na^+]$ range from 0.00001 to 1.1 M.

Data Analysis

Slope conductances. Slope conductances were obtained from current–voltage relationships. The data from membranes with more than one channel were analyzed as follows. When the conductance of channels within a membrane differed by two or more picosiemens, their current transitions were analyzed separately to obtain their corresponding slope conductances. If channels within a membrane had conductance values close to each other (<2 pS), their current transitions were averaged to determine their mean slope conductance.

Voltage dependence of the channel fractional open time (f_o). The voltage dependence of the channel fractional open time (f_o) was obtained by applying 10-mV steps (40–80 s/step) from positive to negative or negative to positive holding potentials (between +70 and −130 mV). Each set of voltage steps is referred to as a voltage run. In the presence of VTD or BTX, the channel's f_o due to closures on the order of milliseconds (fast process) was obtained in the following manner. The total (channels + background) time-averaged conductance was measured by ignoring closures longer than 700 ms. The background conductance was measured during channel closures on the order of seconds and subtracted from the total to obtain the channel time-averaged conductance. Channel f_o was calculated by dividing the channel time-averaged conductance by the number of channels (open during the period measured) and the maximal single channel conductance.

For VTD-modified channels, the channel overall f_o was obtained by including all channel closures. The channel f_o due to closures on the order of seconds (slow process) was obtained by including closures ranging between 0.7 and 20 s; and in both cases data were obtained from membranes with single channels. For a given membrane potential, the channel f_o (overall or that due to the slow process) was obtained by averaging the individual channel f_o values obtained from all performed voltage runs (one to four runs). Subconductance states were counted as the channel being open. The effect of VTD concentrations was studied by measuring the channel's overall f_o while maintaining the membrane potential at −60 mV for 6–138 min.

For BTX-modified channels the actual channel overall f_o (mainly due to long closures in the second and minute range) could not be measured since the number of channels in each membrane could not be determined with certainty and single channel membranes were rare

(see Results). The effect of closures longer than 1 s on the channel overall f_o was obtained in a similar manner as previously described for eel electroplax VTD-modified channels (Duch et al., 1989). At each potential, the time-averaged conductance was measured and normalized with that measured at +40 mV. This measurement will be referred to as the channel's normalized fractional activation instead of the channel's overall f_o.

Serial random analysis. The characterization of the distribution of closures on the order of minutes was done by using serial random analysis (Zar, 1974). Current traces were divided into 20-s intervals. Intervals with channel openings are denoted by n_1 and those without channel openings by n_2. A sequence of like intervals is defined as a run (u). The distribution of runs (u) approaches normality with a mean of:

$$u_u = \frac{2n_1 n_2}{n_1 + n_2} + 1$$

a standard deviation of:

$$s_u = \text{sqrt}\{2n_1 n_2(2n_1 n_2 - n_1 - n_2)/[(n_1 + n_2)^2(n_1 + n_2 - 1)]\}$$

and the statistic of:

$$Z_c = \frac{|u - u_u| - 0.5}{s_u}$$

Z is the normal score, and the normal distribution of a set of normal scores has a mean of zero and a variance of one. In a normalized random distribution the probability of Z being X or larger than X is equal to that of Z being equal to or more negative than $-X$; for example, $P(Z \geq 2) = P(Z \leq -2) = 0.0228$.

TTX block. TTX block was measured as previously described (Levinson, Duch, Urban, and Recio-Pinto, 1986; Duch et al., 1989). After channel incorporation, TTX was added to a final concentration of 30–150 μM to one of the chamber compartments to ensure the study of channels incorporated in only one direction. Each membrane potential was held for 15–30 min and applied in the same manner (for example, changing the membrane potential from 0 to +40 mV) before and after the addition of nanomolar TTX to the other chamber compartment (extracellular aspect of the channels under study). In this manner, slow channel processes (such as long closures and inactivation) should contribute equally to the channel's f_o measured during control (f_o) and after TTX addition (f_{oTTX}). The channel's fractional closed times (f_c) and the apparent binding affinities of TTX ($K_{1/2}$) were calculated as follows:

$$f_c = (f_0 - f_{oTTX})/f_0$$

$$K_{1/2} = [TTX](1/f_c - 1)$$

RESULTS

Veratridine-modified Channels

Current–voltage relationship. Channels from lobster leg nerves were incorporated into neutral planar lipid bilayers in the presence of VTD. In symmetrical 0.5 M NaCl, channel activity was observed in 145 of 329 membranes. Fig. 1 *A* shows current traces from a membrane with a single VTD-modified channel. VTD-modified channels underwent closures on the order of milliseconds, seconds, and minutes even at high positive potentials similar to those reported for other VTD-modified channels (Garber and Miller, 1987; Corbett and Krueger, 1989; Duch et al., 1989). At negative

potentials, when open, VTD-modified channels displayed almost continuously (>98%) their maximal conductance (the largest conductance a channel displays during the recording period). On the other hand, at positive holding potentials, when open, channels displayed continuously their maximal conductance for 1–10 s (Fig. 1 A, arrows) and spent most of the time at a noisy substate; that is, channels opened to a time-averaged conductance level (5.6 ± 0.5 pS, mean ± SEM, $n = 6$ channels) lower than their maximal conductance value (9.6 ± 0.2, mean ± SEM, n = the same 6 channels, 50 Hz). The predominant (displayed by >90% of the channels) maximal channel conductance had a linear and symmetrical current–
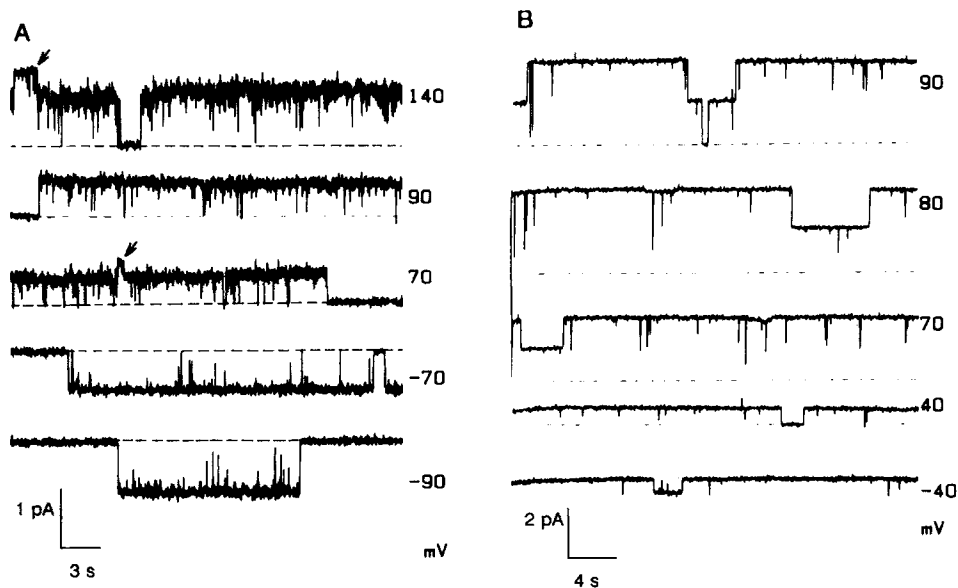

FIGURE 1. VTD- and BTX-modified single channel current transitions in symmetrical 0.5 M NaCl at various membrane potentials. Channel openings are upward at positive potentials and downward at negative potentials. Dashed lines indicate the current level when all the channels are closed. Current traces were filtered at 50 Hz. (A) A membrane with a single VTD-modified channel. At positive potentials the channel remains mostly at a noisy-flickery substate (~6 pS). Arrows indicate when the channel displays its maximal conductance (~10 pS). At negative potentials the channel displays mainly its maximal conductance. (B) A membrane with at least two BTX-modified channels (~16 and 19 pS).

voltage relationship (Fig. 2 inset, filled circles), ranging between 8 and 11 pS (Fig. 2, filled bars) with a mean value of 10 pS. The flickery nature of the noisy substate, with larger spikes toward the fully closed state, suggested the presence of fast, unresolved channel transitions between an open and a nonconducting state. In fact, at high membrane potentials (greater than +100 mV) it was possible to resolve this flickery substate as fast transitions from the fully open to the fully closed state (mean closed time 2–4 ms at 600 Hz). Its predominance at positive potentials initially suggested that it reflected an asymmetry in the channel permeation pathway. However, this is unlikely since some channels could also display this substate at low negative

potentials (0 to −30 mV) and, although infrequently (at 50 Hz), most channels also displayed their maximal conductance at positive potentials. Therefore, this substate does not reflect channel asymmetry but probably a voltage-dependent process consisting of fast closures. Except for its predominance at positive potentials, this substate showed no other voltage dependence, as indicated by its linear, time-
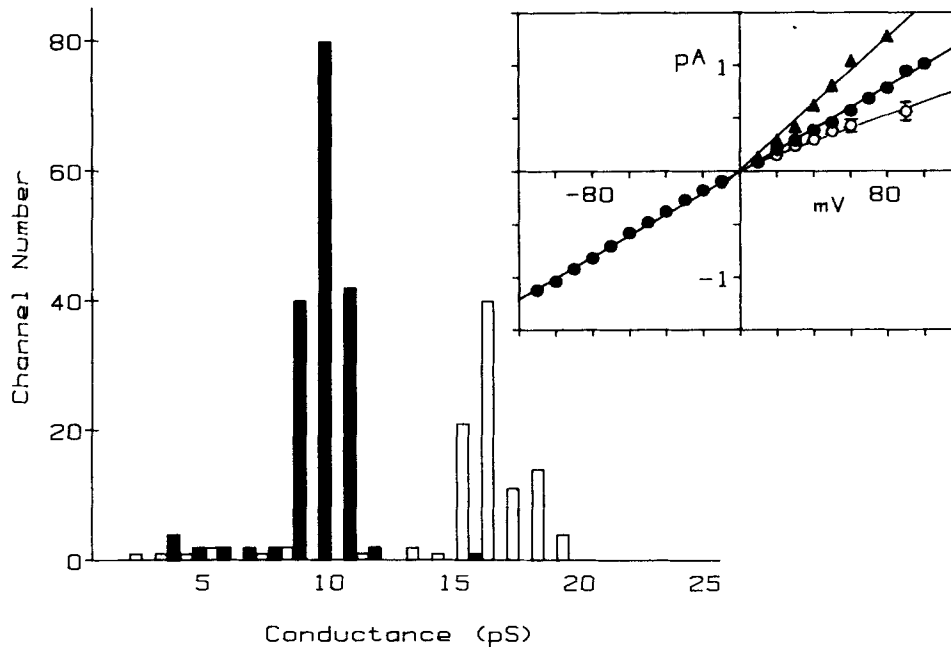

FIGURE 2. Cumulative histograms of single channel maximum slope conductances for alkaloid-modified channels in symmetrical 0.5 M NaCl. Slope conductances were obtained from current–voltage relationships. For BTX-modified channels the shown slope conductances were determined by only using the data at positive holding potentials. This was done because channel openings at negative potentials were observed only in membranes with more than one channel, which made it difficult to determine with certainty whether the current–voltage relationship for single channels was symmetrical or not (see text). *Filled bars*, VTD-modified channels from 86 membranes; *open bars*, BTX-modified channels from 37 membranes. (*Inset*) Current–voltage relationships for the maximal currents of VTD-modified (*filled circles*, 10 pS, 25 channels, 10 membranes) and BTX-modified (*filled triangles*, 16 pS, at least 10 channels, 8 membranes) channels; and for the time-averaged currents during the noisy substate of VTD-modified channels (*open circles*, 6 pS, 18 channels, 9 membranes). Data were fit by linear regression and not forced through 0 mV; most error bars are within symbols.

averaged current–voltage relationship (Fig. 2 inset, open circles). The unresolvable nonconductive state could be either an open-blocked, a closed, or an inactivated state. Since the only cation present in the solution was sodium, the most likely open-blocked state would be channel block by sodium ions. However, this substate showed no apparent dependence on the concentrations of NaCl (Table I).

Voltage-dependent processes. Histograms of channel closings and openings were constructed using data from membranes with single channels (for example, Fig. 3). VTD-modified channels had at least four populations of closed times and two populations of open times. At −60 mV, two of the mean closed times were on the order of milliseconds (26 and 126 ms), one on the order of seconds (2.2 s), and one on the order of minutes (1.2 min) (Fig. 3, bottom left), while the two mean open times were on the order of milliseconds (34 and 861 ms) (Fig. 3, bottom right). Closures on the order of minutes occurred at a rate of about 10 per hour, resulting in a small event histogram peak. However, these long closures were observed in all VTD-modified channels at all holding potentials, and due to their long lifetime they were the major contributors to the channel overall fractional open time (overall f_o) at potentials more positive than −70 mV (see below). Due to the low occurrence of these long closures, data from different channels were pooled together to obtain their

TABLE I

Reduction of the Maximal Channel Conductance of VTD-modified Channels When Going from Their Fully Open (Maximal Conductance) to Their Substate Level

[NaCl]	% of maximal conductance decrease		
	Mean	SEM	n (No. of channels)
mM			
20	37.7	2.8	6
50	35.4	2.9	5
100	30.7	4.2	3
500	40.2	3.5	6

The maximal single channel conductance was measured at positive and negative potentials and the maximal slope-conductance was obtained by fitting a straight line through the data. The time-averaged conductance during the noisy substate was measured at positive potentials and the slope time-averaged conductance was obtained by fitting a straight line through the data. No significant difference was found between any of the groups (nonpaired t test).

mean lifetime. At −60 mV, their closed lifetime ranged between 20 s and 15 min, and had a mean value of 2.81 ± 0.26 min (mean ± SEM, n = 115 transitions from 5 membranes, 120 μM VTD). Their mean lifetime tended to decrease with depolarization; however, it remained in the minute range (1–3 min). This together with event histograms at −50 to −130 mV (three to four single channel membranes per potential) allowed us to separate channel closures into three groups. The first group included all closures shorter than 700 ms, the second group included closures ranging between 1 and 20 s, and the third group included all closures longer than 20 s. We will refer to the closures in the first, second, and third groups as the fast, slow, and very slow channel processes, respectively.

Voltage dependence of the channel's f_o due to the fast process. Fig. 4 A shows that as the membrane potential became more negative, the frequency of channel closures on the order of milliseconds increased. Since the probability of seeing channel openings
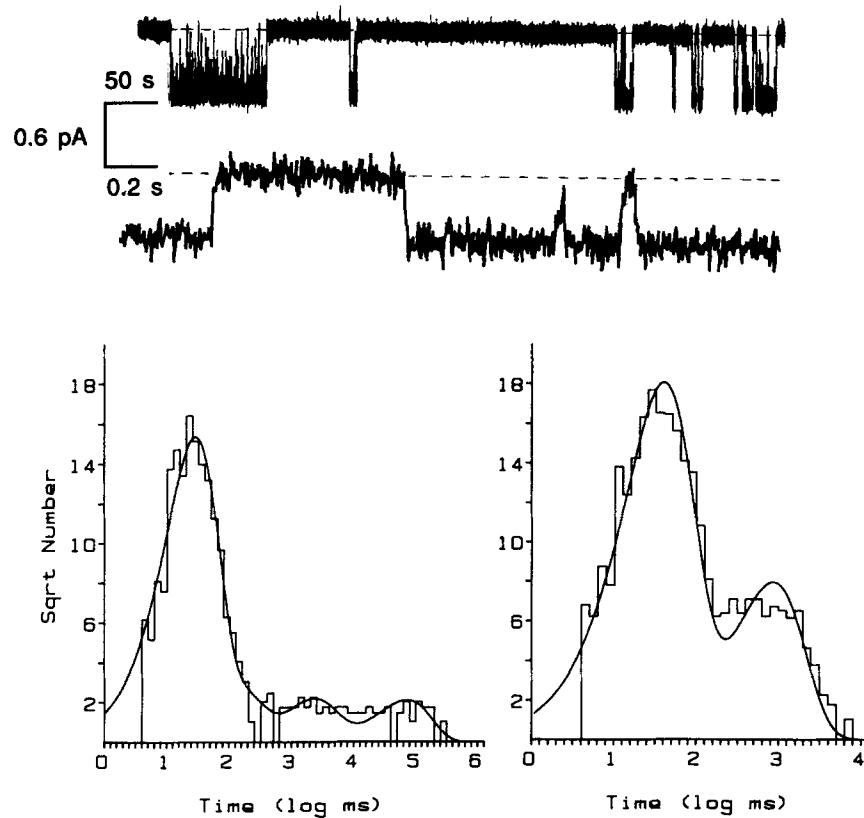
FIGURE 3. Population of channel closings and openings in symmetrical 0.5 M NaCl at −60 mV for a VTD-modified channel. (*Top*) Current traces at −60 mV for a single channel at two time scales filtered at 30 Hz (first trace) and 100 Hz (second trace). Dashed lines indicate the current level when the channel is closed and channel openings are downward. (*Bottom*) Histograms constructed from current traces filtered at 100 Hz and digitized at 1 kHz, ignoring events shorter than 10 ms. *Bottom left*, histogram for closed times showing the presence of at least four populations with time constants of 26 ms, 126 ms, 2.2 s, and 72.2 s (corresponding to 69, 11, 9.7, and 10% of all events, respectively). *Bottom right*, histogram for open times showing the presence of at least two populations with time constants of 37 and 861 ms.

at high negative potentials increased in membranes with more than one channel, data were collected from membranes with one to four channels and averaged to obtain the mean channel fractional open time (f_o) at each potential (see Methods). The mean channel f_o due to the fast process decreased as the potential became more negative (Fig. 4 *B*, open circles). The data were fitted to a two-level Boltzmann distribution (one open and one closed state) (Eq. 1) by using a nonlinear least-squares fit. This was done for simplicity and because the data points represented mean values from various channels.

$$f_o = 1/\{1 + \exp\left[-z_a F(V - V_a)/RT\right]\} \tag{1}$$
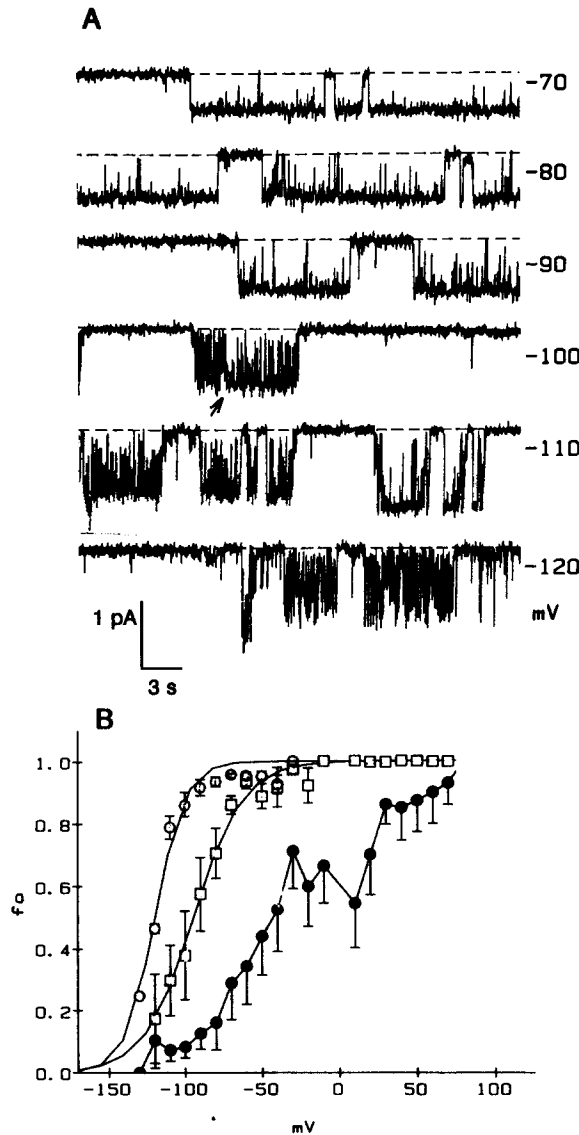
A

B

FIGURE 4. Voltage dependence of VTD-modified sodium channels in symmetrical 0.5 M NaCl. (A) Current traces of a membrane with a single channel at different negative membrane potentials. Dashed lines indicate the current level when the channel is closed and channel openings are downward. Current traces were filtered at 50 Hz. The channel undergoes closures on the order of seconds (slow process) and milliseconds (fast process). At −100 mV the arrow indicates a gating state change (discontinuous change in the channel f_o) for the fast process. (B) Voltage dependence of the channel f_o due to the fast (open circles, 33 channels, 19 membranes), slow (open squares, 13 single channel membranes), and due to all closures (filled circles, 11 single channel membranes). Data points are mean ± SEM. For the fast process, at membrane potentials more positive than −120 mV, the n ranged between 3 and 19, and at more negative potentials the n ranged between 2 and 3. The data for fast and slow processes were fitted to Eq. 1 (smooth solid lines). The fast process had a V_a of −122 mV and a z_a of 2.9; the slow process had a V_a of −95 mV, and a z_a of 1.6.

z_a is the valence of the apparent gating charge, F is the Faraday constant, V is the membrane potential, V_a is the midpoint potential ($f_o = 0.50$), R is the gas constant, and T is the absolute temperature. The fit of the data to Eq. 1 (solid line, Fig. 4 B) gave a population mean V_a of −122 mV and a z_a of 2.9 (33 channels, 19 membranes).

Due to the occurrence of closures on the order of minutes, it was only possible to obtain data points covering the entire voltage range in three membranes with single channels. Their individual V_a and z_a values were first obtained with Eq. 1 and then averaged, resulting in a mean z_a value of 4.1 ± 1.0 (mean ± SEM, $n = 3$ channels) and a mean V_a value of −116 ± 1 mV.

For the fast process, individual channels could display different V_a and z_a values at different times. For example, in a membrane with a single channel during the first voltage run, the channel had a V_a of -112 mV and a z_a of 2.3, while during the second voltage run (14 min later) the channel had a V_a of -121 mV and a z_a of 3.4. Channels could also undergo state changes; that is, changes in their fractional open time while being held at a given membrane potential (for example, Fig. 4 A, arrow at -100 mV). Therefore, the z_a value obtained from the mean channel f_o curve (Fig. 4 B, open circles) will underestimate the population mean z_a value because it was obtained from 33 channels, some of which were observed either to undergo state changes or to change their V_a values with time, or both. Moreover, different channels had different V_a values as suggested by their different f_o values at a given membrane potential (see SEM in Fig. 4 B, open circles).

Slower processes. The channel f_o due to all channel closures (overall f_o) was measured using membranes with single channels by changing the membrane potential from $+70$ to -130 mV (or from -130 to $+70$ mV) progressively in 10-mV steps (40 s/step). When the membrane potential was changed from positive to negative values, the observed voltage dependence of the overall f_o was shallow (Fig. 4 B, filled circles). The channel f_o limited to closures on the order of seconds (slow process) had a midpoint potential of -95 mV and an apparent gating charge of 1.9 (Fig. 4 B, squares). Compared with the overall f_o, the slow process had a sharper voltage dependence and occurred at more hyperpolarized potentials. Compared with the fast process it had a shallower voltage dependence and occurred at more depolarized potentials. The contribution of the fast process to the overall f_o was negligible, since it usually occurred at potentials more negative than -100 mV. The contribution of the slow process was small and primarily at potentials more negative than -70 mV. The overall f_o increased due to an increase in the mean channel open time, which went from the order of milliseconds to minutes as the membrane potential depolarized. The shallowness of the overall f_o resulted from the presence of long closures (minute range) at all membrane potentials.

The overall f_o showed a dependence on the direction of the voltage protocol; that is, the overall f_o values were different when the voltage was applied from positive to negative (Fig. 5; filled circles) than when it was applied from negative to positive (Fig. 5, open circles) values. The largest difference was found at positive potentials where the overall f_o values were higher when potentials were applied from positive to negative. At potentials more negative than -50 mV a smaller difference was found, but in this case the overall f_o values were lower when potentials were applied from positive to negative values. Since channels underwent closures on the order of minutes at all membrane potentials and flickery long openings (second and minute range) at positive potentials, the dependence on the voltage protocol direction in part may reflect that the duration of each voltage step (40 s) was insufficient to provide equilibrium measurements. The effect of using short voltage steps should have been compensated by averaging data from several voltage runs (see Methods) and from several membranes (Fig. 5). However, it is possible that the number of voltage runs was insufficient to provide equilibrium measurements of the very slow process. Another possibility is that in addition to the very slow process there is another voltage-dependent process and its contribution to the overall f_o is the one

that depends on the direction of the voltage protocol. In fact, as described below, the dependence of the overall f_o on the voltage protocol direction appears to result mainly from the presence of an inactivation process that increases with depolarization.

In an attempt to obtain better steady-state measurements for the overall f_o, membrane potentials were held for longer periods (10–100 min). This limited the number of voltages that could be studied in a single membrane; however, it also provided new information. At negative membrane potentials channels continuously underwent openings and closings (Fig. 6 B). However, when the membrane potential was changed from negative to positive values ($\geq$ +40 mV), channels initially underwent long openings (minute range) and up to two long closures (minute range)
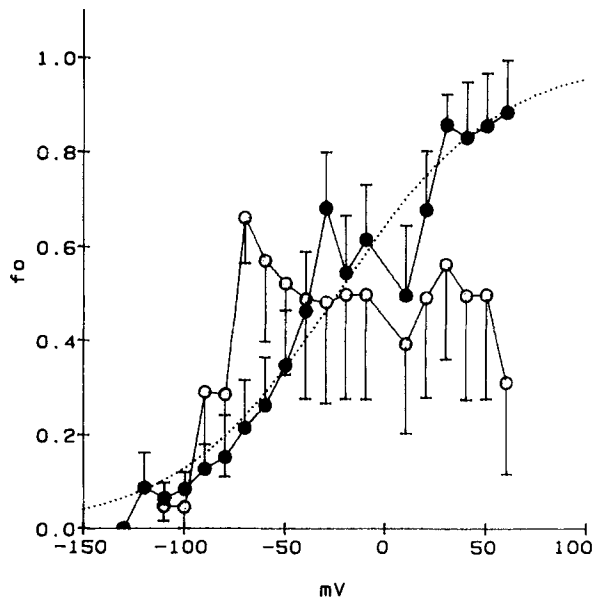

FIGURE 5. The overall f_o dependence on the direction of the voltage protocol. In single channel membranes, the membrane potential was progressively changed in 10-mV steps from +70 to −110 mV (or more negative) (*filled circles*, 6–11 membranes) or from −110 mV (or more negative) to +70 mV (*open circles*, 4–6 membranes). The overall f_o was measured (see Methods) and plotted versus membrane potential. Data points are mean and SEM. The dependence on the voltage protocol direction seems to reflect mainly a higher overlap level with the inactivation process when the voltage protocol was applied from negative to positive (see text). The data obtained when the voltage protocol was applied from positive to negative were fitted to Eq. 1 (*dotted line*) and had a V_a of −24 mV and a z_a of 0.7.

before entering an essentially irreversible nonconductive state (Fig. 6 A). Once a channel entered this nonconductive state, it could be reopened by changing the membrane potential to lower positive ($\leq$ +30 mV) or negative values (Fig. 6 A, second lane). Fig. 7 quantitates the time course of these observations. At −60 mV, the mean overall f_o remained more or less constant with time (Fig. 7, bars), while at +60 mV the mean overall f_o decreased exponentially with a rate constant of 6.7 min, and reached zero at ~20 min (Fig. 7, filled circles). We do not have enough data to describe the voltage dependence of this process; however, it appears that this process becomes stronger (faster current decay) as the potential becomes more positive than +30 mV and decreases as the potential becomes less positive. These results indicate that VTD-modified channels inactivate slowly at high positive potentials. Interest-

ingly, when more than one channel was present in the membrane, the rate of inactivation appeared to be slowed (not shown), probably indicating some type of coordinated behavior between channels. In single channel membranes at +60 mV, 2 of 11 channels underwent long closures before inactivating, whereas the other channels underwent a single long opening and then inactivated. Their mean time before inactivating was 5.15 ± 1.63 min and their mean channel f_o due to only long closures (before inactivation) was 0.909 ± 0.078 (mean $\pm$ SEM, $n = 11$ single channel membranes). These data indicate that the dependence of the overall f_o on the voltage protocol direction shown in Fig. 5 mainly reflects the fact that at positive potentials there was a higher overlap level with the inactivation process when the

A

+60 mV

+60   -60

0.5 pA
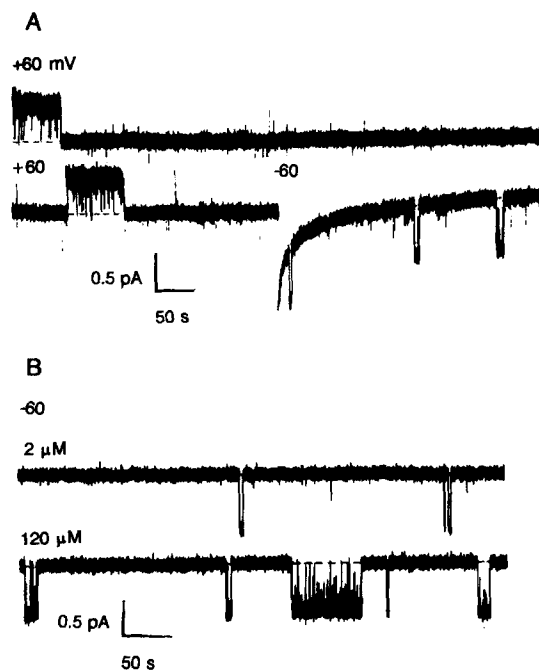
50 s

B

-60

2 µM

120 µM

0.5 pA

50 s

FIGURE 6. Current traces of VTD-modified channels showing the inactivation process and the effect of VTD concentration. Dashed lines indicate the current level when the channel is closed, and channel openings are upward at +60 mV and downward at −60 mV. (*A*) Current traces from two single channel membranes. In both cases the channel underwent long noisy openings before entering a long nonconductive state. The second lane shows how the channel reopens upon changing the potential from +60 to −60 mV. (*B*) Current traces for the same channel at 2 µM VTD (first lane) and 120 µM VTD (second lane). Increasing the VTD concentration increased the burst duration (sequence of channel openings with closures no longer than 20 s) and decreased the interburst duration (see Table III, asterisks).

voltage protocol was changed from negative to positive values, than with the reverse protocol. This is probably the case because before applying the latter protocol (positive to negative) the membrane potential was held at large negative values (−130 to −100 mV), which should remove the inactivation process, and then the membrane potential was changed in one step to a large positive value (usually +70 mV) and the voltage run started. The combination of both removal of inactivation (by initially holding the membrane potential at large negative values) and the slow rate of inactivation of these channels appears to decrease the contribution of the inactivation process to the overall f_o when the voltage protocol was applied from positive to negative potentials. Assuming that this is the case, when the voltage protocol was

applied from positive to negative values, the voltage dependence of the measured overall f_o was predominantly due to the very slow process and could be fitted to Eq. 1, which gave a V_a of -24 mV and a z_a of 0.74 (Fig. 5, dotted line). That is, the very slow process occurred at more depolarized potentials and had a shallower voltage dependence than the fast and slow processes.

We investigated whether closures on the order of minutes (very slow process) reflected unbinding of VTD. This was done by exposing channels to four different VTD concentrations that were known to produce different ^{22}Na fluxes into vesicles
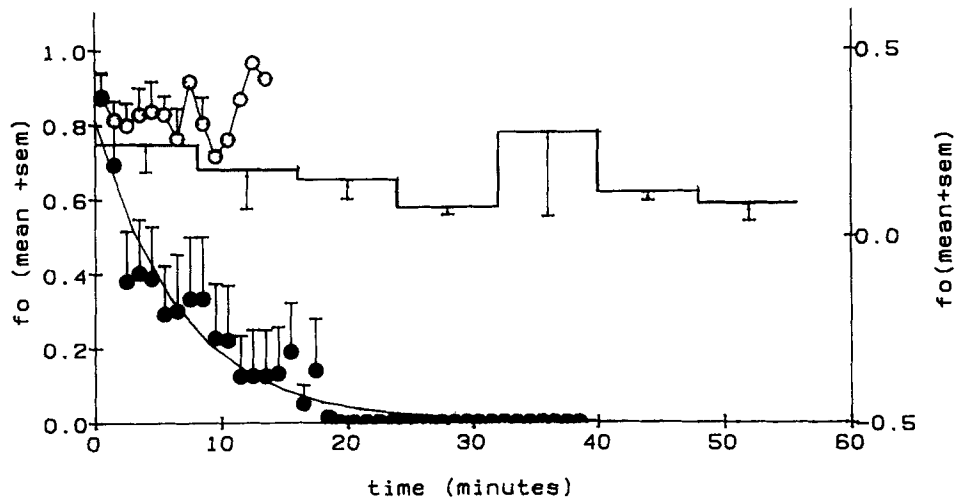

FIGURE 7. Time dependence of the channel overall f_o for alkaloid-modified channels. The overall f_o was averaged every minute at $+60$ mV for VTD-modified channels (*filled circles, left abscissa*) and at high positive potentials ($+60$ to $+90$ mV) for BTX-modified channels (*open circles, right abscissa*); and every 8 min at -60 mV for VTD-modified channels (*stepped line, right abscissa*). Membranes had only one channel in the presence of VTD and one to four channels in the presence of BTX. In the presence of BTX, the channel number in a membrane was estimated by dividing the highest conductance level observed by the single channel conductance. For VTD-modified channels at $+60$ mV, the number of membranes was as follows: 12 (1–6 min); 10 (7 min); 9 (8–12 min); 8 (13–16 min); 7 (17–18 min); 6 (19 min); 4 (20 min); 3 (21–28 min); 2 (31–39 min). For VTD-modified channels at -60 mV, the number of membranes was as follows: 6 (1–16 min); 5 (17–32 min); 4 (33–40 min); and 3 (41–56 min). For BTX-modified channels the number of membranes was as follows: 21 (1 min); 19 (2 min); 14 (3 min); 12 (4 min); 10 (5 and 6 min); 8 (7 min); 6 (8 and 9 min); 5 (10 min); 2 (11–14 min).

containing sodium channels from lobster leg nerves (Correa et al., 1987): 2 μM, which produces less than 5%; 12 μM, which produces just below 50%; and 120 and 500 μM, which produce maximal sodium flux. These experiments were done at -60 mV, since at this potential the major nonconductive state was the very slow one, while the fast, slow, and inactivated processes were either rare or absent and contributed very little to the overall f_o. Thus, at this potential it was possible to study the effect of VTD on the very slow process by measuring the overall f_o even on membranes with more than one channel. The overall f_o of channels from four membranes increased

about fourfold when the VTD concentration was increased from 2 to 120 μM (Table II*A*). Despite the observed channel variability, when values from different membranes were pooled together it was found that VTD still increased the overall f_o in a dose-dependent manner (Table II *B*) without increasing the number of detectable channels. In single channel membranes, it was found that increasing the levels of VTD from 2 to 120 μM increased the membrane mean (M-mean) burst duration (sequence of openings with closures no longer than 20 s) by ~11-fold, while it had no detectable effect on the M-mean interburst duration (Table III). An effect on the interburst duration could have been missed due to our low time resolution (by missing brief channel openings during long closures) and the presence of channel variability. In fact, in the only single channel membrane in which it was possible to study the effect of VTD concentration, there was an increase in the burst duration and a decrease in the interburst duration when the VTD concentration was increased

TABLE II

Effect of VTD Concentrations on the Channel's Overall f_o Expressed as the Percent of Time the Channels Were Found in Their Open State (%t-open)

(A) [VTD]	Obs. time				No. of channels				%t-open					Mean	Sem
	Individual								Individual						
2	50	49	57	88	1	6	3	2	2.6	2.4	3.8	3.3		3.0	0.3
122	55	48	45	48	1	6	3	2	7.8	15.4	5.9	15.8		11.2	2.6

(B) [VTD]	Mean	SEM	Range	Mean	SEM	Range	n
2	37	7	9–57	4.2	1.1	1.3–8.9	7
12	36	8	6–60	7.6	2.8	1.8–16.7	6
122	48	9	11–132	21.3	5.0	7.8–60.0	11
500	12			24.5			1

Observation time is given in minutes. Membrane potential was −60 mV. (*A*) Channels from four membranes were exposed to 2 and 122 μM VTD. After increasing the VTD concentration from 2 to 122 μM the membrane was allowed to equilibrate with VTD for 5–10 min before collecting data. (*B*) Data from *A* together with data from membranes exposed to only one VTD concentration (micromolar). Observation time (Obs. time) is given in minutes, and *n* is the number of membranes.

from 2 to 122 μM (Table III, asterisks; Fig. 6 *B*). These results suggest that the measured overall f_o in part reflects VTD binding. Since long channel closures persisted at maximal VTD concentrations even at positive potentials, we believe that long channel closures probably represent a complex of both short periods (millisecond range) of unmodified states and long periods (minute range) of VTD-modified closed states. Long closures may also represent the time the channels take to cycle through conformations that do not bind VTD; however, this possibility appears less likely (see Discussion).

Even though VTD-modified channels could be observed for long periods, they could also become undetectable during the observation period (membrane lifetime); that is, channels did not reopen for the rest of the observation time, even when the holding potential was changed to various positive or negative values. When channels from a membrane were exposed to a large voltage range (including positive and

negative values) channels from 69% of the membranes (11 of 16) at 2 μM, 44% of the membranes (4 of 9) at 12 μM, and 4% of the membranes (2 of 47) at 120 μM disappeared during the observation time. In contrast, if the membrane potential was kept only at positive values, channels did not disappear during the observation time regardless of the VTD concentration. These results indicate that channels disappeared more readily at negative than at positive potentials. Since channel disappearance occurred without any obvious changes in the lipid membrane characteristics (capacitance, noise), and was dependent on both the membrane potential and VTD concentration, we believe that it reflects VTD unbinding followed by a channel conformational change from one that is capable of binding VTD to one that is not (see Discussion).

TABLE III

Effect of VTD Concentrations on the Channel's Burst (Channel Openings with Closures No Longer Than 20 s) and Interburst Duration

[VTD]	Burst duration			Interburst duration		
	Mean	SEM	n	Mean	SEM	n
μM						
2	*0.15	0.07	12	3.24	0.57	13
	0.07	0.02	3	4.04	3.04	4
	0.08	0.04	4	2.80	1.58	3
M-mean:	0.10			3.36		
12	0.19	0.07	24	2.30	0.26	23
	0.15	0.02	7	4.63	2.55	7
M-mean:	0.17			3.46		
122	*0.41	0.08	22	2.01	0.35	23
	0.84	0.21	19	2.23	0.48	18
	2.34	0.29	5	5.84	1.88	5
	1.68	0.93	9	4.87	0.97	8
	0.39	0.18	11	2.90	0.61	10
M-mean:	1.13			3.57		

Each row shows data from a membrane with a single channel; n is the number of bursts (or interbursts); M-mean is the average of membrane mean values; * denotes data from the same membrane.

Nonrandom distribution of channel openings. The presence of closures on the order of minutes was further examined by modifying channels with supramaximal VTD concentrations (120 μM) and using serial random analysis of two conditions as described under Methods (Zar, 1974), and as previously discussed for the characterization of long channel closures (Horn, Vandenberg, and Lange, 1984). Membrane potentials were held for a period of 11–132 min, and then the current traces were divided into 20-s intervals. The two conditions examined were intervals with channel openings and intervals without channel openings. A sequence of like intervals is defined as a run (u). The chosen interval period (20 s) was long enough to avoid the influence of closures on the order of milliseconds and seconds, and short enough to

characterize the distribution of closures on the order of minutes. In a normalized random distribution, the normal scores are denoted as Z values, the mean value $(\bar{Z})$ is zero, and the variance is one. If trials with channel openings occur together, the number of runs will tend to be smaller than expected for random distribution and Z values will vary from zero (the mean value). If intervals with channel openings never occur together, the number of runs will be large, as expected for a random distribution, and the Z value will be close to zero. Moreover, if channels behave independently, the level of nonrandomness will be reduced as the number of channels present in the membrane increases (Horn et al., 1984). Values of Z were obtained as described under Methods and plotted versus membrane potential (Fig. 8). Almost identical Z values were obtained when using the simplified equation of Horn et al. (1984). The probability of seeing the calculated Z values in a normal distribution was very low, indicating that the distribution of channel openings was nonrandom, because there was a tendency for channel openings to occur together as if they were cycling in and out of a long nonconducting state. At positive potentials, in addition to this tendency, the slow inactivation process also contributed to the nonrandom distribution of channel openings. Moreover, at -60 mV the level of nonrandomness in membranes with more than one channel ($z = 5.3 \pm 2.7$, mean $\pm$ SEM, $n = 4$ membranes with two to six channels) was similar to that in membranes with single channels ($z = 6.6 \pm 0.96$, $n = 7$ membranes). The same appears to occur at other membrane potentials (Fig. 8). This indicates that channels can show coordinated behavior which results in bursts of channel activity.

BTX-modified Channels

In the presence of BTX, channel activity was observed in 70 of 166 membranes. Fig. 1 B shows current traces of a membrane containing more than one BTX-modified channel. BTX-modified channels showed quiet open states (the noise level was the same as background), rarely showed subconductance states, and showed variability (13–18 pS) in their maximal conductance values (Fig. 1 B and 2, open bars). At positive potentials the current–voltage relationship was linear and had a mean conductance value of 16 pS (Fig. 2 inset, filled triangles). At negative potentials, the probability of seeing channel openings was low. In fact, at these potentials channel openings were observed only in membranes with more than one channel. This, in conjunction with the observed variability of single channel conductances (Fig. 1 B), made it difficult to determine with certainty whether the current–voltage relationship for single channels was symmetrical or not. However, the single channel conductance levels observed at negative holding potentials also ranged between 13 and 18 pS.

Voltage-dependent processes. In contrast to other BTX-modified channels, those from lobster leg nerves underwent closures on the order of seconds and minutes, even at high positive potentials (Figs. 1 B and 9). These channels also underwent closures on the order of milliseconds. The characterization of all these processes was limited for the following reasons. First, for experimental reasons, the level of BTX used in most experiments (4–5 μM) was ~10 times lower than the level of BTX known to produce half-maximal flux of sodium ions into vesicles containing sodium channels from lobster leg nerves (Villegas and Villegas, 1981). Second, in most membranes it was not possible to determine the number of channels and there was a
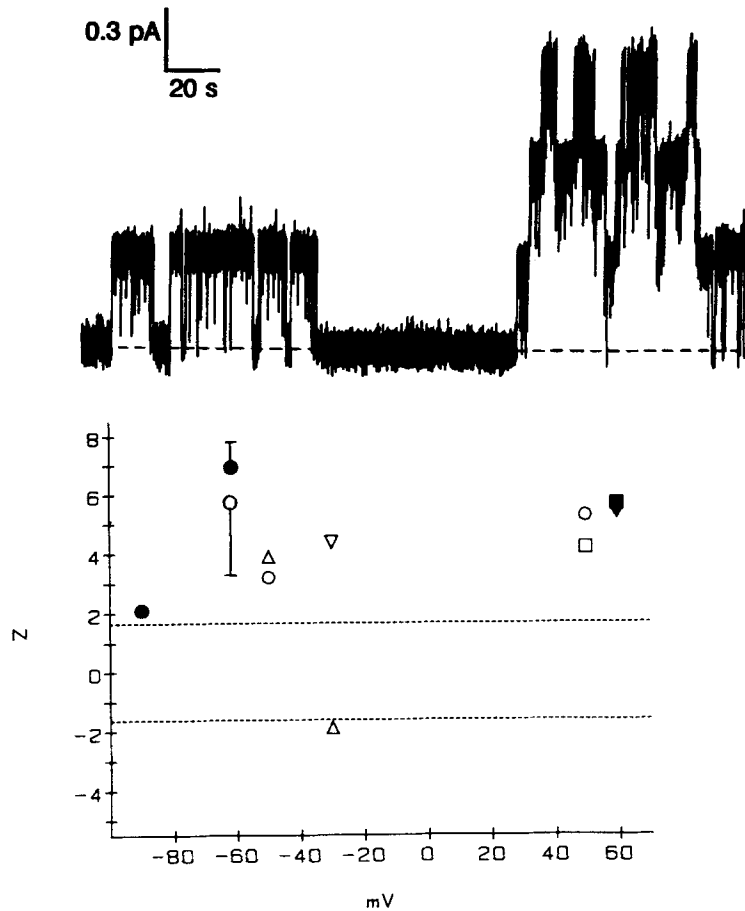
FIGURE 8. Characterization of closures on the order of minutes (very slow process) in symmetrical 0.5 M NaCl. (*Top*) Current record at −50 mV for a membrane with three VTD-modified channels showing channel closures on the order of minutes, seconds, and milliseconds. (*Bottom*) Relationship between randomness of ordering of current record intervals (20 s/interval), with and without channel openings. Random scores (Z values) were obtained as described in Methods and plotted against membrane potential. Filled symbols show data from membranes with single channels, and open symbols show data from membranes with more than one channel. At −60 mV the data represent mean and SEM of membranes with single channels (mean Z = 6.6 ± 0.96, mean ± SEM, n = 7 membranes, range 4.3–10.5), and are similar to those in membranes with two to six channels (mean Z = 5.3 ± 2.7, n = 4 membranes, individual Z values = 2.7, 12.6, 6.1, 0.0). At other membrane potentials, n = 1 membrane and symbols represent different membranes (*open triangles and circles*, two or three channels; *open square*, four channels). The area of the random distribution with 5% significance is Z = ±1.64 and is indicated with dotted lines.

low probability of having membranes with only one channel (only 2–3 out of 70 membranes). Third, channel openings were infrequent at negative holding potentials. Finally, at the concentrations of BTX used (4–5 μM), almost all (>95%) BTX-modified channels disappeared during the observation period and this occurred when large negative potentials (more negative than −40 mV) were applied; that is, channels were not seen again even when positive potentials were reapplied. As for VTD-modified channels, BTX-modified channels did not disappear when the membrane potential was held at positive values.

A

B
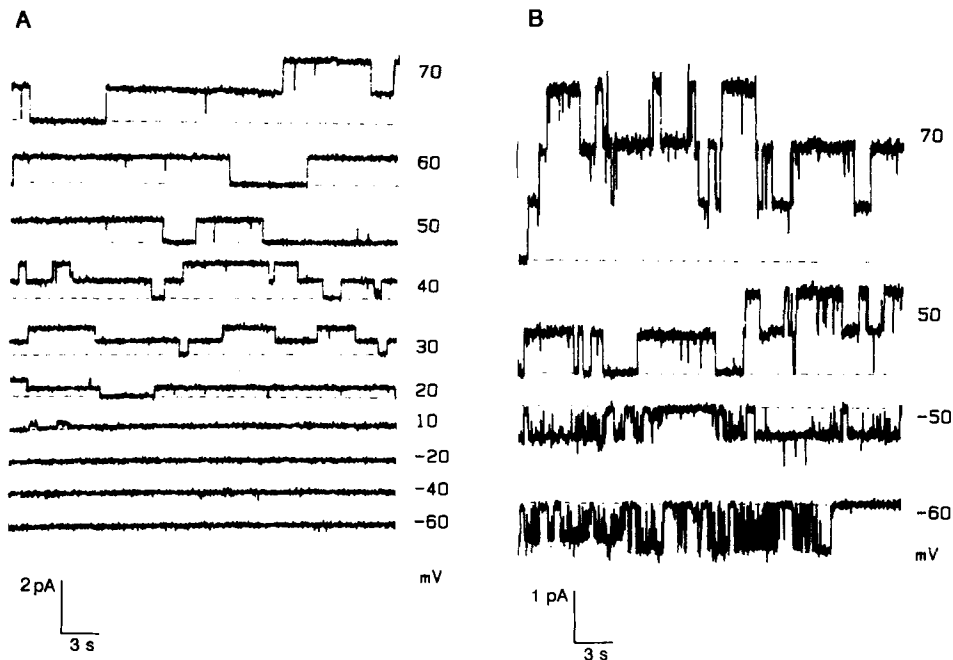
FIGURE 9. BTX-modified single channel current transitions as a function of membrane potential in symmetrical 0.5 M NaCl. Current traces for two membranes, one with at least two (*A*) and the other with at least three (*B*) channels. Current traces were filtered at 50 Hz. Dashed lines indicate the current level when all the channels are closed. Channel openings are upward at positive potentials and downward at negative potentials.

Since in most membranes it was not possible to determine the number of channels, the actual overall f_o due to long closures (> 1 s) could not be obtained. However, an indirect measurement of their effect on the overall f_o was obtained by normalizing the time-averaged conductance at each potential with that at +40 mV (channel fractional activation). The channel fractional activation decreased as the potential became more negative as a result of an increase in the number of long channel closures and a decrease in the number of detectable channels (Fig. 10, filled triangles). As long as the potential was not more negative than −40 mV, channels could usually be reopened upon returning to positive potentials. However, when channels were brought to potentials more negative than −40 mV, the probability of channel

reopening at positive potentials decreased; that is, channels did not reopen during the rest of the observation time (membrane lifetime). We believe that channel disappearance reflects unbinding of BTX; therefore, the channel fractional activation measured from +70 mV to −40 mV results mostly from transitions between BTX-modified conducting and nonconducting states (including closures on the order of minutes), while that determined at more negative potentials may also include unbinding of BTX (channel disappearance). Unlike VTD-modified channels, BTX-modified channels did not show a slow inactivation process when held at high positive potentials (Fig. 7, open circles). Moreover, the low overall f_o value observed at these high positive potentials (Fig. 7, open circles) resulted from the presence of long channel closures (second and minute range).
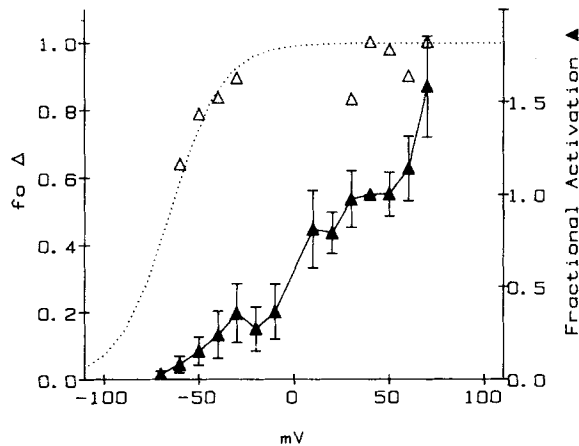

FIGURE 10. Voltage dependence of BTX-modified sodium channels in symmetrical 0.5 M NaCl. Filled triangles represent the mean channel fractional activation obtained by normalizing the time-averaged channel conductance at each potential with that measured at +40 mV. Data points are mean ± SEM for n = 4–13 membranes containing at least 37 channels. Since the actual number of channels for each membrane was unknown, the data were not fitted to any equation but only connected to show the overall trend. Open triangles represent channel f_o due to closures on the order of milliseconds, n = 2 membranes with at least 6 channels. Due to the limited number of data points, the dotted line was drawn (not fitted) by setting the values of V_a = −65 mV and z_a = 1.9 of Eq. 1.

When channel openings occurred at negative membrane potentials, it could be seen that the frequency of closures on the order of milliseconds increased as the membrane potential became more negative (Fig. 9 B). The channel f_o due to closures on the order of milliseconds is shown for channels in two membranes (Fig. 10, open triangles). Channel f_o decreased as the potential became more negative due to an increase in the frequency of these fast closures. Under these conditions, it was not possible to collect data at more negative potentials. However, this process is different (kinetics and the voltage range where it occurs) from that due to long (> 1 s) closures.

Channel Conductance Versus NaCl Concentrations

The single channel conductance of both VTD- and BTX-modified channels was dependent on the concentration of NaCl (Fig. 11). In both cases, the conductance value saturated at high NaCl concentrations, and at low NaCl concentrations it

deviated from a simple Langmuirian isotherm (dotted line, Fig. 11 *A*). That is, as NaCl concentrations approached zero, the conductance value approached a limiting, nonzero value. This is more clearly seen when the data were plotted using an Eadie-Hofstee plot, in which non-Langmuirian isotherms give nonlinear plots (Fig. 11 *B*). At present we cannot eliminate the possibility that the non-Langmuirian
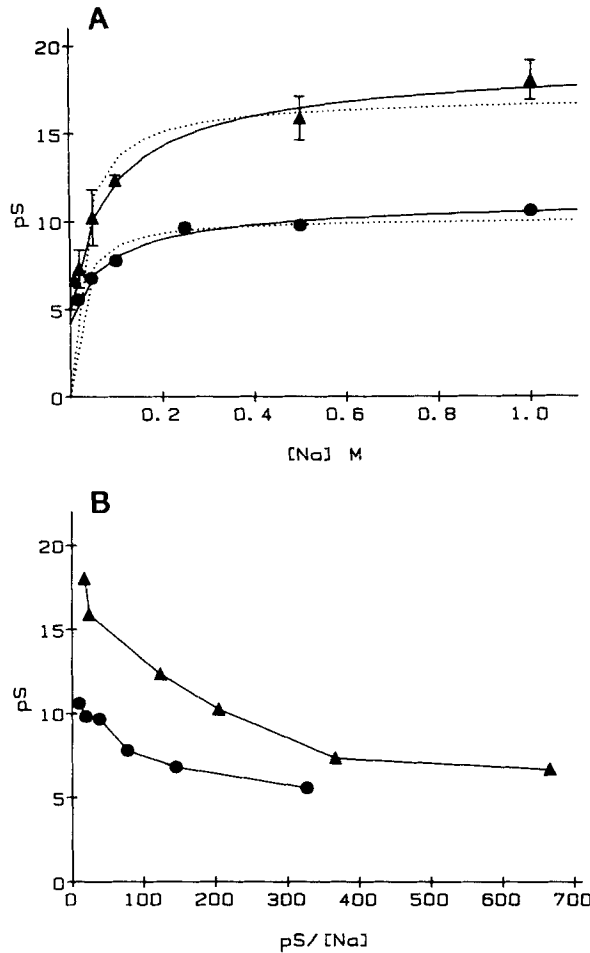

FIGURE 11. Variation of single channel conductance with Na$^+$. (*A*) Single channel conductances of VTD- (*circles*) and BTX- (*triangles*) modified channels plotted against NaCl concentrations. Electrolyte solutions contained the indicated NaCl concentrations without a supporting electrolyte; that is, they have a varying ionic strength. The points denote mean ± SEM. For BTX-modified channels, n = 3–4 membranes with at least 5–8 channels, except for 10 mM NaCl (n = 2, with at least 3 channels) and 500 mM NaCl (n = 24, with at least 76 channels). For VTD-modified channels error bars are within symbols, n = 3–7 membranes, with at least 9–17 channels. Dotted lines are a fit of the data to a Langmuir isotherm ($g = g_{max} \cdot$ [Na$^+$]/(K_{Na} + [Na$^+$])). The fit gave for VTD-modified channels: g_{max} = 10 pS, K_{Na} = 19 mM; and for BTX-modified channels: g_{max} = 17 pS, K_{Na} = 26 mM. Solid lines are a fit of the data to Eq. 4. The fit gave for VTD-modified channels: K_{Na} ≈ 81 mM, g_{max} ≈ 11 pS, and z ≈ −0.08 e/nm^2; and for BTX-modified channels: K_{Na} ≈ 101 mM, g_{max} ≈ 19 pS, and z ≈ −0.07 e/nm^2. (*B*) Eadie-Hofstee plot of the data in *A*. Data points were connected with a solid line. Conductance values were corrected to 22°C using Q_{10} = 1.3.

behavior is in part due to multiple ion occupancy, as has been recently shown for BTX-modified channels from frog skeletal muscle (Naranjo, Alvarez, and Latorre, 1989); nor have we determined whether the sensed surface charge is equal at both channel entrances (Naranjo et al., 1989; Correa, Latorre, and Bezanilla, 1991). Therefore, for comparison purposes, and because it was sufficient to produce a good

fit, the data were fitted to a model that assumes single-ion channel occupancy and an equal distribution of surface charge under symmetrical solutions of NaCl (Green et al., 1987a). In this model, the single channel conductance approaches a limiting value as the NaCl concentrations are increased (saturates) and as the NaCl concentrations approach zero. The latter value is determined by the magnitude of sensed surface charge at the channel's entrance. The presence of a surface charge results in a potential difference (V_e) between the bulk aqueous phase and the entrance of the channel, which can be described by the Gouy-Chapman equation (Eq. 3) (McLaughlin, 1977):

$$V_e = (2 \cdot kT/e) \cdot \text{arcsinh} \left\{ z / (8 \cdot N \cdot [\text{Na}^+] \cdot e_0 e_r \cdot kT)^{0.5} \right\} \tag{3}$$

where z is the apparent charge density at the channel entrance, e_0 is the permittivity of free space, and e_r is the relative dielectric constant of water (79.27 at 22°C; Weast, 1985).

Moreover, with the above assumptions, the surface charge density can be estimated by fitting the data in Fig. 11 to Eq. 4 (Green et al., 1987a):

$$g = g_{\text{max}} \cdot [\text{Na}^+] / \left\{ [\text{Na}^+] + K_{\text{Na}} \cdot \exp \left(V_e \cdot e / kT \right) \right\} \tag{4}$$

where g is the single channel conductance, g_{max} is the maximal conductance, and K_{Na} is the apparent binding affinity for sodium ions. For VTD-modified channels the fit gave a g_{max} of 11 pS, a K_{Na} of 81 mM, and a surface density charge (s) of -0.08 e/nm². For BTX-modified channels the fit gave a g_{max} of 19 pS, a K_{Na} of 101 mM, and a surface density charge of -0.07 e/nm². Although the maximal conductances and apparent binding affinities differ, the sensed surface charge densities were similar for VTD- and BTX-modified channels.

Ion Selectivity of VTD- and BTX-modified Channels

Ion selectivity of alkaloid-modified sodium channels was estimated with reversal potential measurements under mixed ionic and bi-ionic electrolyte conditions. For mixed ionic conditions the chamber compartment where the extracellular aspect of the channel was facing had 810 mM NaCl and the other had 450 mM NaCl and 360 mM KCl (see Methods). Reversal potential measurements for individual (Table IV) and mean (Fig. 12 A, Table IV) membrane values are shown. Under these conditions, VTD-modified channels had a mean reversal potential value of 7.88 ± 0.16 (mean ± SEM, $n = 5$ membranes) and a mean permeability ratio of sodium over potassium ($P_{\text{Na}}/P_{\text{K}}$) of 2.46 ± 0.06 (Fig. 12 A, circles). Similar values were found for BTX-modified channels which had a mean reversal potential value of 7.79 ± 0.21 ($n = 5$ membranes) and a mean $P_{\text{Na}}/P_{\text{K}}$ of 2.48 ± 0.08 (Fig. 12 A, triangles). However, under bi-ionic conditions (cis: 414 mM NaCl; trans: 414 mM KCl) VTD-modified channels had a lower mean $P_{\text{Na}}/P_{\text{K}}$ value than BTX-modified channels (Fig. 12 B). VTD-modified channels had a mean reversal potential of 15.21 ± 1.33 ($n = 9$ membranes) and a mean $P_{\text{Na}}/P_{\text{K}}$ of 1.83 ± 0.11 (circles), while BTX-modified channels had a mean reversal potential of 24.79 ± 2.09 ($n = 5$ membranes) and a mean $P_{\text{Na}}/P_{\text{K}}$ of 2.70 ± 0.23 (triangles). The $P_{\text{Na}}/P_{\text{K}}$ of BTX- but not of VTD-modified channels remained the same whether obtained under bi-ionic or mixed ionic conditions.

TTX Block

Experiments with TTX were done to determine whether or not the observed alkaloid-modified sodium channels were TTX sensitive. High concentrations of TTX (30–150 µM) completely blocked VTD- and BTX-modified channels when added to their extracellular aspect (determined by the voltage dependence of channel f_o). Submaximal concentrations of TTX (approximately nanomolar) blocked the channels in an all or none manner (Figs. 13, top, and 14). VTD-modified channels were blocked with TTX in a voltage-dependent manner with negative potentials increasing block (Fig. 13, bottom). The TTX apparent binding affinity $(K_{1/2})$ at 0 mV was 10.9 nM and the fraction of the applied voltage (a) that affected TTX block was 0.40, which resemble the values reported for VTD-modified channels from purified eel

TABLE IV

Reversal Potentials (V_r) and Permeability Ratios (P_{Na}/P_K) of Alkaloid-Modified Channels under Mixed Ionic Conditions

Toxin	Membrane No.	Na$_o$	K$_o$	Na$_i$	K$_i$	TEA	V_r	P_{Na}/P_K	No. of channels
VTD	1	798	0	438	360	10	7.3	2.46	4
	2	798	0	438	360	10	7.9	2.24	1
	3	814	0	448	364	0	8.0	2.52	3
	4	814	0	448	364	0	8.3	2.62	3
	5	814	0	448	364	0	7.9	2.46	3
							7.88 ± 0.16	2.46 ± 0.06	14
BTX	1	814	0	453	362	5	7.90	2.55	2
	2	810	0	448	364	0	8.02	2.56	2
	3	810	0	448	364	0	7.14	2.23	2
	4	800	0	444	356	25	7.54	2.37	2
	5	796	0	442	354	30	8.37	2.71	3
							7.79 ± 0.21	2.48 ± 0.08	11

The level of the electrolyte solutions facing the extracellular (o) and intracellular (i) aspect of the channels are given in millimolar. When necessary, TEA (millimolar) was added to both compartments to block potassium channel activity. V_r values were interpolated using the data from +30 to −30 mV and given with respect to the chamber containing only NaCl (extracellular aspect of the channel).

electroplax $(K_{1/2} = 17.5$ nM; $a = 0.53$; Duch et al., 1989) and the TTX $K_{1/2}$ for sodium channels from lobster leg nerves determined with flux studies (12 nM; Barnola and Villegas, 1976).

The TTX $K_{1/2}$ at 0 mV for BTX-modified channels was estimated under mixed ionic conditions, in the presence of high concentrations of BTX, and while being held at 0 mV (Fig. 14). These conditions allowed the observation of BTX-modified channels for long periods without undergoing channel disappearance. Nanomolar concentrations of TTX reduced the probability of detecting channel openings (Fig. 14, second trace), while millimolar concentrations of TTX completely blocked channel activity (Fig. 14, third trace). The estimated $K_{1/2}$ at 0 mV was ~84 nM, a value resembling those obtained for other BTX-modified channels when their
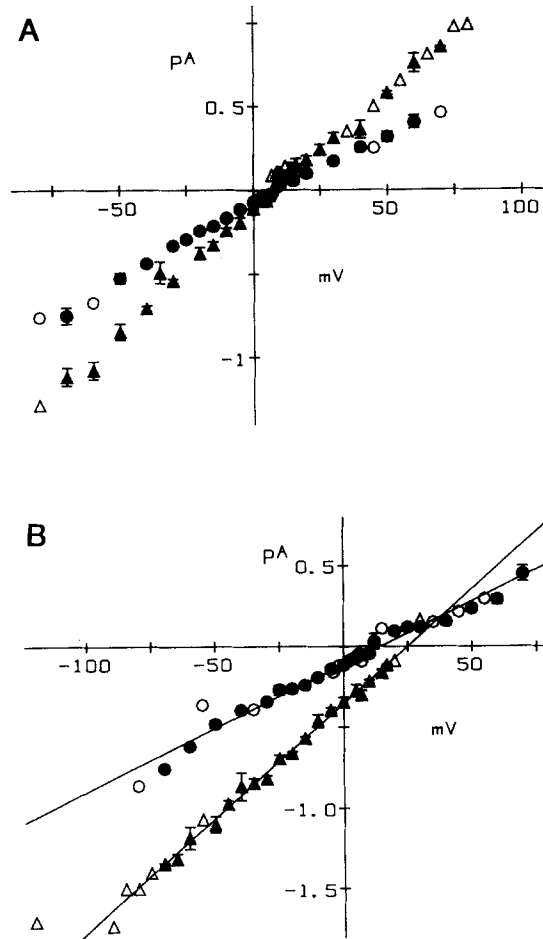
A

pA

0.5

-50   50   100

mV

-1

Δ

B

pA 0.5

-100   -50   50

mV

-1.0

-1.5

Δ   Δ

FIGURE 12. Reversal potential (V_r) measurements of VTD- (circles) and BTX- (triangles) modified channels. V_r values were interpolated using the data from +30 to −30 mV, and the permeability ratio P_{Na}/P_K was calculated using the Goldman-Hodgkin-Katz equation. Open symbols, $n = 1-2$ membranes; filled symbols, $n = 3-9$ membranes. (A) Current–voltage relationships for channels under mixed ionic conditions. The chamber facing the extracellular aspect of the channel contained 808 mM NaCl, and the one facing the intracellular aspect of the channel contained 447 mM NaCl and 361 mM KCl. The holding potential is with respect to the chamber containing only NaCl, which corresponds to the extracellular aspect of the channel. VTD-modified channels had a V_r of 7.88 ± 0.16 and a P_{Na}/P_K of 2.46 ± 0.06 (mean ± SEM, $n = 5$ membranes with at least 14 channels). BTX-modified channels had a V_r of 7.79 ± 0.21 and a P_{Na}/P_K of 2.48 ± 0.08 ($n = 5$ membranes with at least 11 channels). The ionic conditions and V_r and P_{Na}/P_K values for individual membranes are given in Table III. (B) Current–voltage relationships for channels under bi-ionic conditions. One compartment contained 414 mM NaCl and the other 414 mM KCl. Both compartments had 10 mM TEA. In these experiments the channel orientation was not always determined and the given holding potential is with respect to the chamber containing NaCl. VTD-modified channels had a V_r of 15.21 ± 1.33 and a P_{Na}/P_K of 1.83 ± 0.11 ($n = 9$ membranes, at least 26 channels); BTX-modified channels had a V_r of 24.79 ± 2.09 and a P_{Na}/P_K of 2.70 ± 0.23 ($n = 5$ membranes, at least 10 channels). Under bi-ionic conditions the P_{Na}/P_K values of VTD- and BTX-modified channels were significantly different; paired t test).

extracellular aspects are exposed to similar (~800 mM) concentrations of NaCl (Moczydlowski et al., 1984; Green, Weiss, and Andersen, 1987b).

DISCUSSION

In this report TTX-sensitive, voltage-dependent sodium channels from lobster leg nerves were characterized in a well-defined lipid environment. Sodium channel
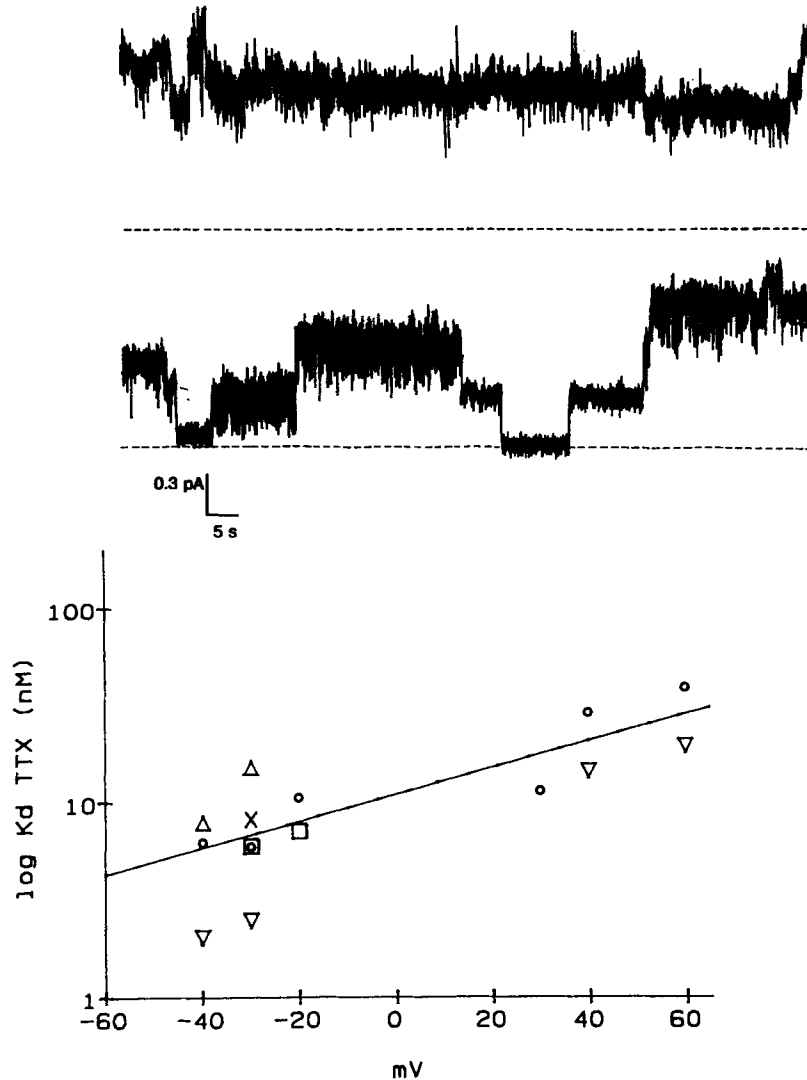
FIGURE 13. Block of VTD-modified channels by TTX in symmetrical 0.5 M NaCl. (*A*) Current traces showing at least three VTD-modified channels of a membrane with seven channels before (top trace) and after (bottom trace) the addition of 15 nM TTX. Holding potential was +40 mV; traces were filtered at 30 Hz. (*B*) Voltage dependence of block by TTX. The TTX level producing 50% of the block ($K_{1/2}$) at each holding potential was calculated as described in Methods and plotted against membrane potential. Symbols represent different membranes (*inverted triangles*, two channels; × and *squares*, three channels; *triangles*, four channels; *circles*, seven channels). The data were fitted using a nonlinear least-squares fit to the equation $K_{1/2}(V)/K_{1/2}(0) = \exp[aFV/(RT)]$; F, Faraday constant; V, membrane potential; R, gas constant; T, absolute temperature; a is the fraction of the applied potential that affects the TTX block. The fit of the data gave a $K_{1/2}(0)$ of 10.9 nM and an a of 0.40.

proteins were incorporated into planar neutral lipid bilayers of lipid composition similar to that used for the study of other sodium channels. The chosen experimental conditions not only permitted comparison of our observations directly with those from other bilayer studies, but also furthered the understanding of how these sodium channels function.

Flickery Open State of VTD-modified Channels

VTD-modified channels at positive holding potentials displayed a noisy substate almost continuously. This substate could also be seen at low negative potentials, indicating that it reflected a voltage-dependent process and not a channel asymmetry (for example, sodium ions being conducted more easily in one direction than in the other). Except for its predominance at positive potentials, this substate showed no
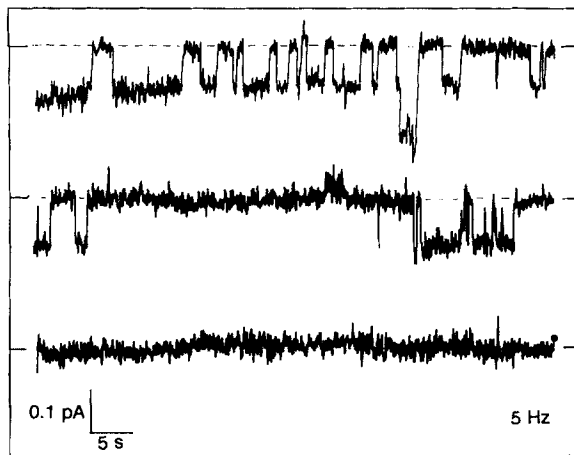
FIGURE 14. Block of BTX-modified channels by TTX in mixed ionic conditions. Channels were first incorporated in symmetrical 0.5 mM NaCl in the presence of 25 μM BTX. Then the ionic conditions were changed to the following: the *cis* compartment, 797 mM NaCl; and the *trans* compartment, 443 mM NaCl, 354 mM KCl, 30 mM TTX. Both compartments had 30 mM TEA. The P_{Na}/P_K was estimated (see membrane 5 in Table III). Afterward, the membrane potential was held at 0 mV before the addition of TTX (32 min, top trace), after the addition of 153 nM TTX (15 min, middle trace), and after the addition of 30 mM TTX (30 min, bottom trace) to the *cis* compartment. Current traces were filtered at 5 Hz. Channel openings are downward, and dashed lines indicate the current level when all channels are closed. In the presence of 153 nM TTX the estimated $K_{1/2}(0)$ for TTX was 84 nM.

other voltage dependence. We looked into the possibility that the noisy substate represented block by sodium ions, but the substate showed no dependence on the sodium concentration. This tendency of VTD-modified channels to remain at a noisy substate while being held at positive potentials has been described for purified channels from rat brain (Corbett and Krueger, 1989) but not for those from skeletal muscle (Garber and Miller, 1987; Corbett and Krueger, 1989). Furthermore, VTD-modified channels from eel electroplax show variability with respect to this behavior: most channels displayed their maximal conductance, but a small amount (<1%) displayed a noisy-flickery state (Duch et al., 1989). The last preparation contains sodium channels mainly from electroplax cells which are muscle derived, but probably also from nerve endings since electroplax cells are innervated. Whether the presence of various cell membranes accounts for the observed functional variability in

the latter preparation still needs to be determined. However, it appears that the flickery noisy substate of VTD-modified channels is predominant in neuronal sodium channels, while it is rare in channels derived from skeletal muscle.

Voltage Dependence of the Channel's Fractional Open Time

The kinetics and voltage dependence of the channel's f_o due to closures on the order of milliseconds (fast process) resemble the previously described voltage-dependent activation of single BTX-modified sodium channels in planar bilayers (Moczydlowski et al., 1984; Hartshorne et al., 1985; French et al., 1986b; Recio-Pinto et al., 1987; Cukierman, Zinkand, French, and Krueger, 1988; Chabala, Urban, Weiss, Green, and Andersen, 1991). Although this process has not been previously described in bilayer studies for VTD-modified channels, its voltage dependence (V_a, z_a) resembles that described in biological membranes for the fast developing macroscopic VTD-modified currents (Leibowitz et al., 1986; Sutro, 1986; Rando, 1989). Moreover, the kinetics and sharp sigmoidal shape, but not the V_a value, resemble those of VTD-modified channels from neuroblastoma cells studied with patch clamp (Barnes and Hille, 1988). In the latter case, the different V_a values may reflect differences between channel fast activation properties or between channel lipid environments. As in other sodium channel preparations (Khodorov, 1985; Strichartz, Rando, and Wang, 1987), this fast activation process occurred at more negative potentials in VTD-modified channels than in BTX-modified channels. The channel's f_o, due solely to closures on the order of seconds, increased relatively sharply with depolarization, and it may contribute to the voltage-dependent activation of VTD-modified channels. From these data, it cannot be determined whether the fast and slow processes represent the same activation process undergoing "state-changes," or separate activation processes. Interestingly, in some neurons the presence of two activation processes has been reported, one of which belongs to a persistent small sodium current that is responsible for their repetitive firing characteristics (Dubois and Bergman, 1975; French and Gage, 1985; French, Sah, Buckett, and Gage, 1990). It will be interesting to determine whether the repetitive firing of lobster leg nerves (Wright, 1958) is due also to sodium currents, and if so how it can be related to the present observations.

The shallow voltage dependence of the overall f_o resembled that reported for other VTD-modified sodium channels studied in planar bilayers (Corbett and Krueger, 1989; Duch et al., 1989) and for the slow developing, macroscopic, VTD-modified currents studied in voltage-clamped frog nodes of Ranvier (Rando, 1989). Moreover, the midpoint potential value was close to that reported for VTD-modified channels from rat brain (Corbett and Krueger, 1989). In the case of lobster nerve VTD-modified channels, the increase in the overall f_o with depolarization was due to an increase in the mean channel open time, while the shallowness of its voltage dependence resulted from the presence of closures on the order of minutes. Since measurements were done in single channel membranes, neither of these effects (increase in the overall f_o, and shallowness) resulted from an increase in the number of detectable channels. In contrast, an increase in the number of detectable channels seems to be a factor for both of these measurements in VTD-modified channels from eel electroplax (Duch et al., 1989). The voltage protocol used in these studies (10-mV

steps, 40 s/step) was chosen for comparison purposes with other studies using VTD-modified channels. At least in lobster VTD-modified channels the dependence on the voltage protocol direction mainly reflects a different level of overlap with an inactivation process; however, it may also reflect nonequilibrium measurements.

Increasing the VTD concentration increased the overall f_o and the channel's burst duration, and probably (as indicated by the result in one single channel membrane) reduced the interburst duration. However, closures on the order of minutes persisted at super-saturating VTD concentrations. Long closures may represent either a combination of VTD-modified closed and unmodified channel states or the time channels take to cycle through conformations that do not bind VTD. The latter possibility is unlikely, since if true one would expect to continuously observe a channel after its incorporation into the bilayer. However, channels disappeared and the rate of channel disappearance was higher at low VTD concentrations and also increased with hyperpolarization. Based on these observations, we believe that channel disappearance results from VTD unbinding followed by a change in channel conformation from one that can bind VTD to one that cannot bind VTD. When a channel disappeared, it could not be reopened, either by changing the membrane potential to positive or negative values, or by increasing the VTD concentration. The former indicates that the channel assumes conformations that do not bind VTD at negative and positive potentials, while the latter suggests that the unmodified channel spends very little time on conformations that bind VTD and most of the time on conformations that do not bind VTD.

In membranes where the VTD concentration was increased from 2 to 120 μM, the number of detectable channels did not increase. Interestingly, similar increases of the VTD concentration did increase the number of detectable skeletal muscle sodium channels in planar bilayers (Garber and Miller, 1987), probably reflecting a difference between sodium channels from both sources. One explanation could be that most (if not all) of the unmodified states of skeletal muscle sodium channels are capable of binding VTD, while only a few of those of lobster nerve sodium channels are capable of binding VTD. This would also explain why when muscle sodium channels were studied, the bilayer membranes always had more than one VTD-modified channel (Garber and Miller, 1987). We believe that long closures consist of several channel states: long VTD-modified closed states and unmodified states that are capable of binding VTD. These unmodified states probably include both nonconducting and conducting states that are too brief to be resolved in the bilayer. If this is the case, the time the channels expend on these unmodified states should decrease as the VTD concentration is increased. However, this effect would usually be undetectable because the lifetimes of the unmodified states are brief (millisecond range) compared with that of the long VTD-modified closed state (minute range). Any apparent reduction of the lifetime of long closures may result from decreasing the number of events that consist of multiple long VTD-modified closed states (separated by brief unmodified states) and increasing the number of events that consist of only one long VTD-modified closed state. The large increase in the burst duration may result from the difference in VTD binding affinities between various unmodified channel states. It has been shown in other systems that the channel state with the highest VTD binding affinity is the open state (Sutro, 1986; Barnes and

Hille, 1988; Rando, 1989). Therefore, it is possible that at the low VTD concentrations used (2 μM), the VTD binding rate to the unmodified open state(s) was close to maximal, while that of the unmodified nonconducting state(s) was submaximal. Then, increasing the VTD concentration to 120 μM would have affected mainly the VTD binding rate to the nonconducting state(s). This would have increased the probability of transitions occurring from the unmodified to the VTD-modified closed state, and from the latter to the VTD-modified open state. In turn, this would have increased the burst duration and the channel overall f_o without a need for a large decrease in the interburst duration. In the case of VTD-modified channels from skeletal muscle, increasing the VTD concentration would have an additional effect; that is, it would increase the number of channels in the bilayer that become modified (and hence detected) with VTD. Therefore, the overall f_o for lobster VTD-modified channels seems to reflect mainly the equilibrium between VTD-modified open states and a long VTD-modified closed state. The contribution due to VTD binding would be small because unmodified states were relatively very brief, and the probability for modifying other sodium channels (present in the bilayer) with VTD was essentially zero (since the number of detectable VTD-modified channels did not increase when the VTD concentration was increased). In the case of channels such as those from skeletal muscle, the contribution of VTD binding to the overall f_o would be large, since all unmodified states (hence all unmodified channels present in the bilayer) seem capable of binding VTD, as suggested by the reported increase in the number of detectable VTD-modified channels when the VTD concentration was increased (Garber and Miller, 1987).

The observation that channel openings tended to cluster (burst-like behavior), even when more than one channel was present in the membrane, indicates that channels are capable of interacting with each other. Another indication of interaction between channels is the apparent slowing of the channel inactivation process in membranes with more than one channel. In native membranes there is evidence for coordinated openings of BTX-modified channels from hybrid neuroblastoma cells (Iwasa, Ehrenstein, Moran, and Jia, 1986). But this type of behavior would be unexpected in planar bilayers, since one would expect that after channel incorporation individual channel proteins will diffuse away from each other and therefore show independent behavior. However, it is possible that these channels interact strongly through their channel structures (e.g., the channel's lipid domain) or through their immediate surroundings (e.g., membrane lipids or other native membrane proteins).

In contrast to VTD, the level of BTX required to observe a reasonable amount of channel activity was higher (4–5 μM) than that used in other bilayer studies. This observation is consistent with the higher concentrations of BTX required to produce 50% maximum sodium flux in vesicles containing sodium channels from lobster leg nerves (Villegas and Villegas, 1981) than in vesicles containing channels from other tissues (Rosenberg et al., 1984; Tamkun et al., 1984; Tanaka et al., 1986; Duch and Levinson, 1987a). Modification with BTX appeared to be reversible, as suggested by the relatively high rate of channel disappearance. At this concentration of BTX, almost every observed BTX-modified channel (>95%) disappeared. The rate of channel disappearance increased with hyperpolarization; that is, channels would not reopen even when potentials were changed back to positive values. This is in contrast

to what has been found for other sodium channels to which BTX binds essentially irreversibly. The reversibility of BTX binding appears to reflect differences between channel structures and not between lipid environments, since the lipid environment used in this study was the same as that used in other planar bilayer studies. BTX binding reversibility is not a general characteristic of sodium channels from peripheral nerves since those from frog node of Ranvier (Dubois et al., 1983) and from squid axons (Correa et al., 1991; Tanguy and Yeh, 1991) bind BTX almost irreversibly.

Permeation

The conductance versus NaCl relationship of VTD- and BTX-modified sodium channels from lobster leg nerves was non-Langmuirian at low NaCl concentrations. That is, their conductance was higher than expected for Langmuirian behavior. In contrast, the conductances of VTD-modified channels from rat skeletal muscle have Langmuirian behavior (Garber and Miller, 1987). With respect to BTX-modified channels, the observed deviation from Langmuirian behavior was lower than that reported for channels from dog brain (Green et al., 1987a) and frog muscle (Naranjo et al., 1989). Moreover, BTX-modified sodium channels from rat brain (French et al., 1986a), rat skeletal muscle (Garber and Miller, 1987), and squid optic nerve (Behrens et al., 1989) show Langmuirian behavior. Non-Langmuiran behavior may result from the surface charge sensed by the permeation pathway, or by multiple-ion channel occupancy. Most previous studies have assumed that alkaloid-modified channels were single-ion occupied (Moczydlowski et al., 1984; French et al., 1986a; Garber and Miller, 1987; Green et al., 1987; Garber, 1988; Behrens et al., 1989). However, more recent evidence indicates that BTX-modified channels from frog muscle are occupied by more than one ion, even though the data still require a surface charge component to obtain a good fit (Naranjo et al., 1989). Assuming that all sodium channels characterized in neutral lipid bilayers have the same level of ion occupancy, then the magnitude of the sensed surface charge changes with sodium channel type (Green et al., 1987; Naranjo et al., 1989). Moreover, for a given tissue the sensed surface charge seems to be the same during VTD and BTX modifications (Fig. 11; Garber and Miller, 1987). Whether or not the conformational changes a channel undergoes during alkaloid modification affect the distribution of the surface charge sensed by the permeation pore in a similar manner still needs to be determined. Recently it has been shown that BTX does not change the surface charge density sensed by the permeation pore of sodium channels from squid giant axon (Correa et al., 1991). However, as discussed in Correa et al. (1991), the surface charge sensed by the permeation pore of sodium channels from squid axons may originate from the channel's structure or the charged native lipids, or from both.

The values of the permeability ratio of sodium to potassium (P_{Na}/P_K) found in this study are lower than those found for other alkaloid-modified channels (approximately four to five) in planar bilayers (see Table V in Recio-Pinto et al., 1987; Garber, 1988; Duch et al., 1989). Moreover, the P_{Na}/P_K value of BTX- but not VTD-modified channels was the same whether obtained under bi-ionic or mixed ionic conditions. Under bi-ionic conditions channel orientation was not determined; therefore, we do not know whether the different P_{Na}/P_K values found for VTD-modified channels

(under bi-ionic and mixed ionic conditions) reflect channel asymmetry (Garber, 1988) or multiple-ion channel occupancy (Naranjo et al., 1989).

Two Types of Channels Based on Their Alkaloid Responsiveness

The level of alkaloid toxin reported to produce 50% of the maximum sodium flux in vesicles containing sodium channels from lobster leg nerves is ~ 20 μM for VTD (Correa et al., 1987) and 50 μM for BTX (Villegas and Villegas, 1981). The value for VTD is similar to, and the one for BTX is 50–300 times larger than, the values found when using vesicles containing sodium channels from other sources (Rosenberg et al., 1984; Tamkun et al., 1984; Tanaka et al., 1986; Duch and Levinson, 1987a). The basis for the low apparent binding affinity (and apparent high binding reversibility) of BTX to these channels is not clear. One possibility is that the alkaloid binding site is the same in the various channels, but the channels differ in the type of conformational changes they undergo upon alkaloid binding. Another possibility is that the alkaloid binding site itself differs between channels in such a manner that BTX, but not VTD, is highly sensitive to these structural differences. Regardless of the mechanism, it can be said that based on alkaloid responsiveness there are at least two types of sodium channels, one type that binds BTX with high affinity and almost irreversibly, and another type that binds BTX with low affinity and with an apparent high reversibility (as suggested by the channel disappearance). Both channel types bind VTD with similar affinity.

So far functional studies using planar bilayers indicate that in brain and muscle tissue there are sodium channels that bind BTX with high affinity. In those studies it would have been difficult to detect channels that bind BTX with low affinity (if present) since the presence of channels with high affinity for BTX would have obscured their detection, and because the concentrations of BTX used (nanomolar) would have bounded preferentially to channels with high but not low BTX binding affinity. Therefore, it would be of interest to perform experiments investigating whether there are sodium channels that bind BTX with low affinity in brain and muscle tissues. Variability in the alkaloid responsiveness may introduce another level of channel functional regulation that awaits the isolation of endogenous factors that could modulate sodium channel activity by binding to their alkaloid binding site.

REFERENCES

Barnes, S., and B. Hille. 1988. Veratridine modifies open sodium channels. *Journal of General Physiology*. 91:421–443.

Barnola, F. V., and R. Villegas. 1976. Sodium flux through the sodium channels of axon membrane fragments isolated from lobster nerves. *Journal of General Physiology*. 67:81–90.

Behrens, M. I., A. Oberhauser, F. Bezanilla, and R. Latorre. 1989. Batrachotoxin-modified sodium channels from squid optic nerve in planar bilayers. Ion conduction and gating properties. *Journal of General Physiology*. 93:23–41.

Chabala, L. D., B. W. Urban, L. B. Weiss, W. N. Green, and O. S. Andersen. 1991. Steady-state gating of batrachotoxin-modified sodium channels. Variability and electrolyte-dependent modulation. *Journal of General Physiology*. 98:197–224.

Corbett, A. M., and B. K. Krueger. 1989. Polypeptide neurotoxins modify gating and apparent single-channel conductance of veratridine-activated sodium channels in planar lipid bilayers. *Journal of Membrane Biology*. 110:199–207.

Correa, A. M., R. Latorre, and F. Bezanilla. 1991. Ion permeation in normal and batrachotoxin-modified Na^+ channels in the squid giant axon. *Journal of General Physiology*. 97:605–625.

Correa, A. M., G. M. Villegas, and R. Villegas. 1987. Anemone toxin II receptor site of the lobster nerve sodium channel. *Biochimica et Biophysica Acta*. 897:406–422.

Cukierman, S., W. C. Zinkand, R. J. French, and B. K. Krueger. 1988. Effects of membrane surface charge and calcium on the gating of rat brain sodium channels in planar bilayers. *Journal of General Physiology*. 92:431–447.

Dubois, J. M., and C. Bergman. 1975. Late sodium current in the node of Ranvier. *Pflügers Archiv*. 357:145–148.

Dubois, J. M., M. F. Schneider, and B. I. Khodorov. 1983. Voltage dependence of intramembrane charge movement and conductance activation of batrachotoxin-modified sodium channels in frog node of Ranvier. *Journal of General Physiology*. 81:829–844.

Duch, D. S., and S. R. Levinson. 1987a. Neurotoxin-modulated uptake of sodium by highly purified preparations of the electroplax tetrodotoxin-binding glycopeptide reconstituted into lipid vesicles. *Journal of Membrane Biology*. 98:43–55.

Duch, D. S., and S. R. Levinson. 1987b. Spontaneous opening at zero membrane potential of sodium channels from eel electroplax reconstituted into lipid vesicles. *Journal of Membrane Biology*. 98:57–68.

Duch, D. S., E. Recio-Pinto, C. Frenkel, S. R. Levinson, and B. W. Urban. 1989. Veratridine modification of the purified sodium channel alpha-polypeptide from eel electroplax. *Journal of General Physiology*. 94:813–831.

Duch, D. S., E. Recio-Pinto, C. Frenkel, and B. W. Urban. 1988. Human brain sodium channels in bilayers. *Molecular Brain Research*. 4:171–177.

Feller, D. J., J. A. Talvenheimo, and W. A. Catterall. 1985. The sodium channel from rat brain. Reconstitution of voltage-dependent scorpion toxin binding in vesicles of defined lipid composition. *Journal of General Physiology*. 260:11542–11547.

French, C. R., and P. W. Gage. 1985. A threshold sodium current in pyramidal cells in rat hippocampus. *Neuroscience Letters*. 56:289–293.

French, R. J., B. K. Krueger, and J. F. Worley. 1986a. From brain to bilayer: sodium channels from rat neurons incorporated into planar lipid membranes. *In* Ionic Channels in Cells and Model Systems. R. Latorre, editor. Plenum Publishing Corp., New York. 273–290.

French, C. R., P. Sah, K. J. Buckett, and P. W. Gage. 1990. A voltage-dependent persistent sodium current in mammalian hippocampal neurons. *Journal of General Physiology*. 95:1139–1157.

French, R. J., J. F. Worley III, M. B. Blaustein, W. O. Romine, Jr., K. K. Tam, and B. K. Krueger. 1986*b*. Gating of batrachotoxin-activated sodium channels in lipid bilayers. *In* Ion Channel Reconstitution. C. Miller, editor. Plenum Publishing Corp., New York. 363–383.

Garber, S. S. 1988. Symmetry and asymmetry of permeation through toxin-modified Na^+ channels. *Biophysical Journal.* 54:767–776.

Garber, S. S., and C. Miller. 1987. Single Na^+ channels activated by veratridine and batrachotoxin. *Journal of General Physiology.* 89:459–480.

Gilly, W. F., and C. M. Armstrong. 1984. Threshold channels: a novel type of sodium channel in squid giant axon. *Nature.* 309:448–450.

Green, W. N., L. B. Weiss, and O. S. Andersen. 1987*a*. Batrachotoxin-modified sodium channels in planar lipid bilayers. Ion permeation and block. *Journal of General Physiology.* 89:841–872.

Green, W. N., L. B. Weiss, and O. S. Andersen. 1987*b*. Batrachotoxin-modified sodium channels in planar lipid bilayers. Characterization of saxitoxin- and tetrodotoxin-induced channel closures. *Journal of General Physiology.* 89:873–903.

Hartshorne, R. P., B. U. Keller, J. A. Talvenheimo, W. A. Catterall, and M. Montal. 1985. Functional reconstitution of the purified brain sodium channel in planar lipid bilayers. *Proceedings of the National Academy of Sciences, USA.* 82:240–244.

Horn, R., C. A. Vandenberg, and K. Lange. 1984. Statistical analysis of single sodium channels. Effects of N-bromoacetamide. *Biophysical Journal.* 45:323–335.

Huang, L.-Y. M., N. Moran, and G. Ehrenstein. 1982. Batrachotoxin modifies the gating kinetics of sodium channels in internally perfused neuroblastoma cells. *Proceedings of the National Academy of Sciences, USA.* 79:2082–2085.

Iwasa, K., G. Ehrenstein, N. Moran, and M. Jia. 1986. Evidence for interactions between batrachotoxin-modified channels in hybrid neuroblastoma cells. *Biophysical Journal.* 50:531–537.

Khodorov, B. I. 1985. Batrachotoxin as a tool to study voltage-sensitive sodium channels, of excitable membranes. *Progress in Biophysics and Molecular Biology.* 45:57–148.

Khodorov, B. I., and S. V. Revenko. 1979. Further analysis of the mechanisms of action of batrachotoxin on the membrane of myelinated nerve. *Neuroscience.* 4:1315–1330.

Kirsch, G. E., and A. M. Brown. 1989. Kinetic properties of single sodium channels in rat heart and rat brain. *Journal of General Physiology.* 93:85–99.

Leibowitz, M. D., J. B. Sutro, and B. Hille. 1986. Voltage-dependent gating of veratridine-modified Na channels. *Journal of General Physiology.* 87:25–46.

Levinson, S. R., D. S. Duch, B. W. Urban, and E. Recio-Pinto. 1986. The sodium channel from Electrophorus electricus. *Annals of the New York Academy of Sciences.* 479:162–178.

Levinson, S. R., W. R. Thornhill, D. S. Duch, E. Recio-Pinto, and B. W. Urban. 1990. The role of nonprotein domains in the function and synthesis of voltage-gated sodium channels. *In* Ion Channels. Vol. 2. T. Narahashi, editor. Plenum Publishing Corp., New York. 33–64.

McLaughlin, S. 1977. Electrostatic potentials at the membrane-solution interfaces. *Current Topics in Membranes and Transport.* 9:71–144.

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

Naranjo, D., O. Alvarez, and R. Latorre. 1989. Ion conduction characteristics of batrachotoxin-modified channels from frog muscle. *Biophysical Journal.* 55:402*a*. (Abstr.)

Patlak, J. B., and M. Ortiz. 1989. Kinetic diversity of Na^+ channel bursts in frog skeletal muscle. *Journal of General Physiology.* 94:279–301.

Rando, T. A. 1989. Rapid and slow gating of veratridine-modified sodium channels in frog myelinated nerve. *Journal of General Physiology.* 93:43–65.

Recio-Pinto, E., D. S. Duch, S. R. Levinson, and B. W. Urban. 1987. Purified and unpurified sodium channels from eel electroplax in planar lipid bilayers. *Journal of General Physiology.* 90:375–395.

Recio-Pinto, E., W. B. Thornhill, D. S. Duch, S. R. Levinson, and B. W. Urban. 1990. Neuraminidase treatment modifies the function of Electroplax sodium channels in planar lipid bilayers. *Neuron.* 5:675–684.

Rosenberg, R. L., S. A. Tomiko, and W. S. Agnew. 1984. Reconstitution of neurotoxin-modulated ion transport by the voltage-regulated sodium channel isolated from the electroplax of Electrophorus electricus. *Proceedings of the National Academy of Sciences, USA.* 81:1239–1243.

Sherman, S. J., and W. A. Catterall. 1985. The developmental regulation of TTX-sensitive sodium channels in rat skeletal muscle in vivo and in vitro. *In* Regulation and Development of Membrane Transport Processes. J. S. Graves, editor. John Wiley & Sons, Inc., New York. 237–265.

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

Stühmer, W., F. Conti, H. Suzuki, X. Wang, M. Noda, N. Yahagi, H. Kudo, and S. Numa. 1989. Structural parts involved in activation and inactivation of the sodium channel. *Nature.* 339:597–603.

Sutro, J. B. 1986. Kinetics of veratridine action on Na channels of skeletal muscle. *Journal of General Physiology.* 87:1–24.

Tamkun, M. M., J. A. Talvenheimo, and W. A. Catterall. 1984. The sodium channel from rat brain. Reconstitution of neurotoxin activated ion flux and scorpion toxin binding from purified components. *Journal of Biological Chemistry.* 259:1676–1688.

Tanaka, J. C., R. E. Furman, and R. L. Barchi. 1986. Skeletal muscle sodium channels: isolation and Reconstitution. *In* Ion Channel Reconstitution. C. Miller, editor. Plenum Publishing Corp., New York. 277–305.

Tanguy, J., and J. Z. Yeh. 1991. BTX modification of Na channels in squid axons. I. State dependence of BTX action. *Journal of General Physiology.* 97:499–519.

Ulbricht, W. 1972. Rate of veratridine action on the nodal membrane. II. Fast and slow phase determined with periodic impulses in the voltage clamp. *Pflügers Archiv.* 336:201–212.

Villegas, R., F. Sorais-Landaez, J. M. Rodriguez-Grille, and G. M. Villegas. 1988. The lobster nerve sodium channel: solubilization and purification of the tetrodotoxin receptor protein. *Biochimica et Biophysica Acta.* 941:150–156.

Villegas, R., and G. M. Villegas. 1981. Nerve sodium channel incorporation in vesicles. *Annual Review of Biophysics and Bioenergetics.* 10:387–419.

Weast, R. C. 1985. CRC Handbook of Chemistry and Physics. CRS Press, Inc., Boca Raton FL. E-49.

Wright, E. B. 1958. The effect of low temperatures on single crustacean motor nerve fibers. *Journal of Cellular and Comparative Physiology.* 51:29–65.

Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., Englewood Cliffs, NJ. 307.