VOLTAGE-DEPENDENT BLOCK BY SAXITOXIN OF SODIUM CHANNELS INCORPORATED INTO PLANAR LIPID BILAYERS

ROBERT J. FRENCH, JENNINGS F. WORLEY, III, AND BRUCE K. KRUEGER
Departments of Biophysics and of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

ABSTRACT We have previously studied single, voltage-dependent, saxitoxin-(STX) blockable sodium channels from rat brain in planar lipid bilayers, and found that channel block by STX was voltage-dependent. Here we describe the effect of voltage on the degree of block and on the kinetics of the blocking reaction. From their voltage dependence and kinetics, it was possible to distinguish single-channel current fluctuations due to blocking and unblocking of the channels by STX from those caused by intrinsic channel gating. The use of batrachotoxin (BTX) to inhibit sodium-channel inactivation allowed recordings of stationary fluctuations over extended periods of time. In a range of membrane potentials where the channels were open >98% of the time, STX block was voltage-dependent, provided sufficient time was allowed to reach a steady state. Hyperpolarizing potentials favored block. Both association (blocking) and dissociation (unblocking) rate constants were voltage-dependent. The equilibrium dissociation constants computed from the association and dissociation rate constants for STX block were about the same as those determined from the steady-state fractional reduction in current. The steepness of the voltage dependence was consistent with the divalent toxin sensing 30–40% of the transmembrane potential.

INTRODUCTION

Recently, we reported that single sodium channels from rat brain could be incorporated into planar lipid bilayers (Krueger et al., 1983). In the presence of the neurotoxin batrachotoxin (BTX), unitary current fluctuations resulting from the opening and closing of individual channels were observed. The reconstituted sodium channels, closed at hyperpolarizing potentials, were selective for sodium over potassium or cesium, and were blocked by nanomolar concentrations of STX. In that study we found that block of sodium channels by STX was voltage-dependent, with hyperpolarizing potentials favoring block. Here we present a more detailed analysis of this voltage dependence.

An accurate description of the effect of voltage on the toxin-induced block is an essential step toward understanding the molecular details of the toxin's action. The voltage dependence could be due to the passage of the divalent toxin through part of the transmembrane voltage as it approaches or leaves its binding site. Although our data are consistent with this simple picture, the actual physical basis of the voltage dependence could be more complex. For example, an obligatory voltage-dependent reaction, such as a gating transition or the vacating of a nearby ionic binding site, could be associated with toxin binding. Evaluation of the voltage dependence under a variety of conditions will be needed to choose among the different alternatives. These data should permit determination of the position, within the transmembrane field, of the toxin binding site, or identification of the nature of an associated voltage-dependent reaction.

Because sodium channels normally inactivate within a few milliseconds after opening, peak sodium currents during depolarizing steps from hyperpolarized holding potentials are normally used to determine channel availability for conduction. Voltage-dependent block by TTX or STX would be missed if such a voltage-clamp method were used to study the degree of block. Because of the slow association and dissociation rates for STX and TTX binding, a relaxation time of several seconds, at nanomolar concentrations, is expected for a new steady-state block to be reached. Thus, the decrease in peak sodium current during a single test pulse indicates the degree of block at the holding potential, because the block cannot re-equilibrate within the duration of the test pulse. The use of BTX, which inhibits sodium channel inactivation (Albuquerque et al., 1976; Khodorov, 1978; Huang et al., 1982), has allowed us to study the voltage dependence of block by STX in the steady state.

MATERIALS AND METHODS

Planar Bilayer Formation

Planar bilayers were formed by the technique of Mueller et al. (1963) across a hole (0.25 mm diam) in a polystyrene partition separating solutions containing 0.5 M NaCl, 0.15 mM $CaCl_2$, 0.1 mM $MgCl_2$, 0.05

mM EGTA, 10 mM Na-HEPES, pH 7.0. The membrane-forming solution contained 33 mg/ml phosphatidylethanolamine (bovine brain) and 13 mg/ml phosphatidylserine (bovine brain) in decane. The phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL); decane was repurified before use by passage over neutral alumina. Saxitoxin (paralytic shellfish poison standard) was obtained from the Food and Drug Administration (Cincinnati, OH); BTX was a gift from Dr. John Daly (NIAMDD, Bethesda, MD).

Preparation of Membrane Vesicles

Membrane vesicles were prepared from rat brain as described by Krueger et al. (1979). Briefly, rat forebrains were homogenized in isotonic sucrose using a cavitating tissue disrupter (Ultraturrax, Tekmar Co., Cincinnati. OH) and the homogenate was subjected to differential centrifugation. The 1,000 *g* and the 10,000 *g* pellets were discarded and the 100,000 *g* pellet (P_3) was resuspended in 0.4 M sucrose at 10–20 mg protein/ml. Aliquots of P_3 were stored at −77° for up to six months without loss of activity. This fraction was enriched about fivefold in ^{3}H-STX binding sites as compared to the crude brain homogenate (Krueger et al., 1979).

Incorporation of Sodium Channels

Incorporation of sodium channels was accomplished using the "fusion" technique (Miller, 1978; Cohen et al. 1980; 1982). The membrane vesicle suspension (100–500 ng/ml protein) was added to the *cis* side of a preformed planar bilayer and BTX (600 nM) was added to the *trans* side. The conductance of the membrane increased in steps, each reflecting the incorporation of one to several single sodium channels (Krueger et al., 1983). Following incorporation of the desired number of channels (normally one to five), the *cis* side was perfused with vesicle-free solution. STX was added to the *cis* side from a 1,000-fold concentrated stock solution.

Data Acquisition and Analysis

The current across the bilayer was measured and command voltages applied to the bathing solutions via a pair of Ag/AgCl electrodes. The side to which brain membrane vesicles were added was designated the *cis* side; the opposite (*trans*) side was held at virtual ground. Prior to addition of biological material, the conductance of the bilayer was <20 pS (~10^{-8} S/cm^2) and the capacitance was ~0.5 μF/cm^2. Two current-to-voltage converters were used in these studies, one having a bandwith of ~80 Hz and the other ~500 Hz. Similar results for the steady-state voltage dependence of gating of BTX-activated sodium channels, in the absence of STX, were obtained with each of these converters. The faster amplifier was built using an LF157H operational amplifier (National Semiconductor, Santa Clara, CA), and a 10^9 (± 1%) Ω feedback resistor (Eltec model 102). Data were recorded continously using an FM tape recorder (Vetter, model B) during an experiment, at or near the bandwidth of the current-to-voltage converter. Subsequently, the data were digitized in 4,096 point-segments at 1, 2 or 5 ms per point using a Nicolet 2090-3A digital oscilloscope, and the digitized records were stored on floppy disks. Data transcription and analysis were controlled by a microcomputer (Micro II, Plessey Peripheral Systems, Irvine, CA) based on an LSI-11/2 processor (Digital Equipment Corporation, Maynard, MA). Software for data handling and analysis was developed using the interpretive language of DAOS (Data Analysis Operating System, Laboratory Software Associates, Melbourne, Australia).

For analysis of STX-induced current fluctuations, the records were filtered (low-pass) with a corner frequency of 30 Hz using an eight-pole Bessel filter (902 LPF 1B, Frequency Devices, Haverhill, MA). This eliminated most of the brief closing fluctuations caused by the voltage-dependent channel gating process, leaving visible the longer events which were primarily due to STX block and unblock. Even at a bandwidth of 500 Hz, many of the visible transitions resulting from the voltage-dependent channel gating were incompletely resolved. We have not attempted a dwell-time analysis of these fluctuations.

All records were visually monitored on a CRT display during analysis. Limiting current levels used in determining the fractional open times, and discriminator levels for the dwell-time analyses, were checked against a whole group of records before carrying out the analysis. If necessary, the selected values were changed to accommodate small shifts in the baseline within a series of records. Also, all analyses were oriented toward the interval, usually the whole record, demarcated by two vertical cursors. When necessary, we excluded from the analysis abnormally noisy segments of records—for example, occasional periods of high frequency, nonquantal fluctuations possibly caused by partial, reversible, breakdown of the membrane.

Fractional open times, resulting from voltage-dependent channel gating and from STX block of the channels, were determined independent of the dwell-time analysis. The background leakage current, i_L, across the membrane, with all incorporated channels closed or blocked, was determined either by setting a horizontal cursor at the low conductance edge of the envelope of these fluctuation, or by averaging cursor-selected, long, well-defined events during which no channels were conducting. The maximum current, i_{max}, (all channels conducting) was determined in an analogous manner. The average current, $\langle i \rangle$, over a long stretch of record (averaging ~20 s for gating, or ~170 s when studying STX-block) was determined. The fractional open time, f_o, is then given by

$$f_o = (\langle i \rangle - i_L)/(i_{max} - i_L) \quad (1)$$

and the fractional closed or fractional blocked time is $(1 - f_o)$.

Dwell-time distributions were determined, one conductance level at a time, by scanning records for periods when the current fell in a target zone between two discriminator levels. These discriminator levels were centered between the conductance level of interest and the adjacent ones on either side. The program was based on a digital triggering routine which recognized positive or negative slope transitions into or out of the target zone. Dwell times were scored as a density function that recorded the number of occurrences at each possible dwell time. After scanning a complete set of records, cummulative distribution functions were calculated (see Fig. 7) to display the total number of events lasting at least as long as any given time, t. If a conductance level corresponds to a single, time-homogeneous, Markovian kinetic state (e.g., Colquhoun and Hawkes, 1981; French and Horn, 1983), these distributions should be a single exponential with a mean value equal to the time constant for decay of the exponential. In fact, for 19 calculated distributions, based on an average of 126 events each, the time constants, τ, were linearly dependent on the mean dwell times, $\langle t \rangle$ (correlation coefficient, $r = 0.97$) with a slope of 1.3. Thus, the distributions were approximately, but not perfectly, exponential, with more short events than expected. These additional events may have been due to the transient, partial breakdown of the membrane already mentioned, or to some infrequently occupied, poorly resolved, and short-lived kinetic state. We cannot choose between these alternatives. In the Results and Discussion sections that follow, we define the characteristic dwell-time constant, τ, for a distribution as the value obtained by a linear least-squares fit of the cumulative distribution to the function

$$\ln N(t) = \ln N_T - t/\tau \quad (2)$$

where $N(t)$ is the number of events with a lifetime of at least t, and N_T is the total number of observations. The fits were performed over the decline of the distributions from N_T to a value of ~0.05 N_T. (For each of the 19 log-transformed cumulative distributions, $r > 0.97$.)

Voltage Convention

In this paper we have expressed all voltages in the normal cellular physiological convention (inside minus outside) on the assumption that STX acts only from the extracellular side. This is the reverse of the convention, $E_m = E(cis) - E(trans)$, used in a number of planar bilayer publications and by Krueger et al. (1983).

RESULTS

Single-Channel Current Fluctuations Due to Gating and Due to STX Block

Fig. 1 illustrates the two temporally distinct populations of current fluctuations through sodium channels in bilayers when both BTX and STX are present. The first of these consists of brief closing events that generally last for periods of a few tens of milliseconds or less, and appears to include many events that are too brief to be completely resolved at the bandwidth of our recordings (cf. Quandt and Narahashi, 1982). When viewed at sufficiently high resolution, many other fluctuations are seen as roughly square current steps, which consistently correspond to a unit conductance of ~30 pS in 500 mM NaCl (see Fig. 3). This population of brief closures, from a well-defined "open" current level, can be seen in both the absence and the presence of STX (see upper and lower records in Fig. 1). We presume that these relatively rapid fluctuations are due to the voltage-dependent gating of the BTX-activated sodium channels. The steady-state voltage dependence will be discussed in more detail below. Observations on numerous preparations appear to show a slight but systematic variation in the kinetics of the fluctuations when the voltage was changed from −60 mV to +60 mV. At positive potentials, closings were slightly less frequent, but tended to be somewhat longer in duration. However, over this entire range, the channels remained open almost all (>98%) of the time. This point will become important in our analysis of the voltage dependence of STX block.

Examining the lower section of Fig. 1, it is immediately obvious that a number of "closing" events of much longer duration appear after addition of STX. In fact, "closing" of the channels becomes much more probable and, at −60 mV, there are several intervals during which neither channel is in the conducting state. These long periods during which one or both channels are "closed" occur only in the presence of STX, and hence it is reasonable to conclude that they are caused by STX block of the channels rather than by closing of the channel gates. This conclusion is also supported by the observation that the probability of entering one of these long-lived "closed" states increases proportionately as the STX concentration is increased (Krueger et al., 1983, and see below).

Fig. 1 illustrates two further points. At +60 mV, with 1 nM STX present, no particularly long-lived "closing" step occurs. Immediately after the voltage change to −60 mV, ~30 s follow in which there is still no extended "closure." Both channels remain open for most of this time. At longer times, however, there are continuous stochastic current fluctuations including periods during which both, one, or neither channel is blocked.[1] (Only the 0-channel and 1-channel levels are shown after the initial blocking event in Fig. 1, lower trace.) Thus, the lower record in Fig. 1 hints at two conclusions: that negative voltages favor block by STX, and that it takes many seconds to establish a new stationary-state following a step in voltage at this STX concentration.

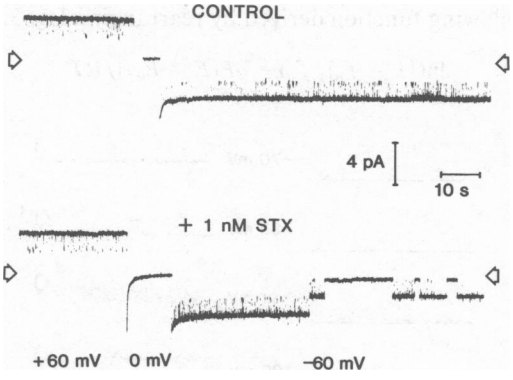

FIGURE 1 Current fluctuations from a membrane containing two BTX-activated sodium channels. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. Zero current levels are shown by the arrows. *Top trace*: control record. The channels remained open almost all of the time, with brief closings towards the zero-current level. These closings were presumably due to the voltage-dependent gating of the channels. The +60 mV segment was actually recorded after the −60 mV segment, but is shown here preceding the −60 mV segment to facilitate comparison with the lower record taken in the presence of STX. Capacity transients are limited by the range of the pen sweep. *Lower trace:* record taken after addition of 1 nM STX to the *cis* side. The long closing steps, including an extended period during which neither channel was conducting, were most likely due to block of individual channels resulting from the binding of STX. Records were filtered (low pass) at 60 Hz on playback.

Dependence of the Open and Blocked Dwell Times on STX Concentration

If we are to establish that the slower population of fluctuations, just described, are caused by STX binding to and dissociating from the channels, two criteria should be met. First, the lifetime of the blocked state should depend only on the intrinsic rate of dissociation of the blocker from the channel, and hence should be independent of STX concentration. Second, for any given channel, the lifetime of the open state will depend on the rate of binding of the toxin, which, for a 1:1 stoichiometry, would be directly proportional to the STX concentration. This prediction may be generalized to apply to a membrane containing several channels using the birth-death analysis applied by Labarca et al., (1980). With the additional assumption that channels present in the membrane are blocked independently of

[1] In Fig. 1 (bottom trace) the time spent at the two-channel level (neither channel blocked) prior to the long "closure" after changing to −60 mV is about equal to the mean dwell time (28 s) predicted by the rate constant for STX block determined later in the paper. The calculated probability that both channels would remain unblocked for at least 30 s following the voltage step to −60 mV is $p = 0.34$.

one another, one predicts the following relations

$$1/\tau_o = n\lambda \tag{3}$$

where τ_o represents the characteristic dwell-time constant from the distribution of "zero-channel–open" dwell times, n is the number of channels in the membrane ($n = 7$ for the experiment that was analyzed in detail for this paper), and λ is the rate of dissociation of STX from a single channel (in s^{-1}). In addition,

$$1/\tau_k = (n - k)\lambda + k\beta[\text{STX}]. \tag{4}$$

Here, τ_k is the characteristic dwell time in the current or conductance level for which k channels are open, and β is the rate constant, in $M^{-1}\ s^{-1}$, for binding of STX to the channel. For the one-channel level, as one approaches conditions under which all channels are blocked, $1/\tau_1$ approaches β[STX].

In Fig. 2, we plot the characteristic reciprocal dwell times against [STX] at +58 mV, for the zero- and one-channel levels. The value of $1/\tau_1$ increases linearly with [STX] (slope = $1.1 \times 10^6\ M^{-1}\ s^{-1}$), while on the same scale, $1/\tau_o$ is essentially independent of STX concentration ($r = -0.28$). The zero intercepts of the regression lines do not differ significantly from the predictions of Eqs. 3 and 4, given the scatter in the data. Thus, the fluctuation kinetics are consistent with the commonly accepted 1:1 stoichiometry for STX binding to the channels.

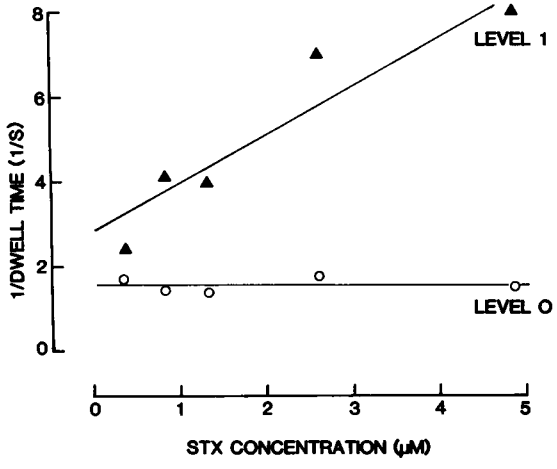

FIGURE 2 Dependence of the reciprocal dwell times on the STX concentration. Dwell times at each concentration were estimated as described in Materials and Methods. The reciprocal dwell time in level 0 (no channels open) is determined by the rate of STX unbinding from the channel, and is independent of STX concentration. The reciprocal dwell time in level 1 (one channel conducting) is primarily determined by the rate of STX binding, and is linearly dependent on STX concentration. Lines shown are linear least-square fits to the data. For level 1, slope = $1.1 \times 10^6\ M^{-1}s^{-1}$ and the correlation coefficient, $r = 0.93$. For level 0, $r = -0.28$. $E = +58$ mV.

Voltage-dependence of Gating of BTX-activated Sodium Channels

The data presented in Figs. 3 and 4 show how a channel's intrinsic, voltage-dependent gating determines the probability that a channel will be found in the open state. In the sample current records at −70 mV (Fig. 3, right), presented on a time scale expanded about 20-fold with respect to those of Fig. 1, one can see only a few closing fluctuations in the current record. Although at −70 mV most are too brief to be completely resolved, many more closing fluctuations are visible as the voltage is further hyperpolarized to −80 mV, and at −90 mV the channel flickers rapidly between open and closed states, spending an almost equal fraction of the time in each. At −100 mV the channel is closed ~3/4 of the time. A parallel illustration of this voltage-dependence appears in the frequency-amplitude histograms, in which the number of data points at each conductance level is plotted against conductance.

In Fig. 4, we have defined the operational range and steepness of this voltage-dependence by fitting the fractional open time, f_o, to a Boltzmann function of the voltage, E:

$$f_o = 1/\{1 + \exp(qF[E - E_{0.5}]/RT)\}. \tag{5}$$

The precise values taken by this function are determined by the parameters q, the apparent gating charge, and $E_{0.5}$, the voltage at which $f_o = 0.5$. The value of q determines the slope of the curve and $E_{0.5}$ is the midpoint of the range of voltages over which f_o changes. Actual values of these parameters were obtained by a linear least-squares fit to the following function derived by rearranging Eq. 5.

$$\ln([1 - f_o]/f_o) = qF(E - E_{0.5})/RT. \tag{6}$$

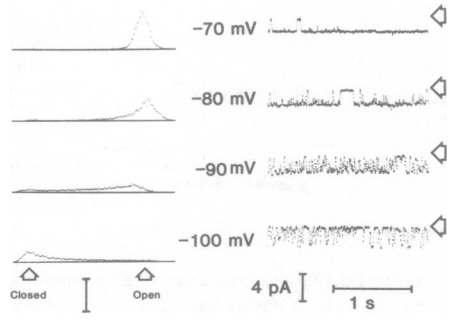

FIGURE 3 Voltage dependence of gating of a single, BTX-activated sodium channel. Histograms of frequency of occurrence vs. conductance (*left*), and sample digitized records (*right*) are shown. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. The arrow on the right side of each current record indicates the zero-current level. The vertical bar at the bottom of the *left* panel indicates 500 points for the histograms; the spacing between the arrows below the histograms is 30 pS. Records were filtered at 80 Hz on playback from an FM recorder. In the range of −70 mV to −100 mV, the channel goes from spending most of the time in the open state (−70 mV) to spending about 3/4 of the time in the closed state (−100 mV).

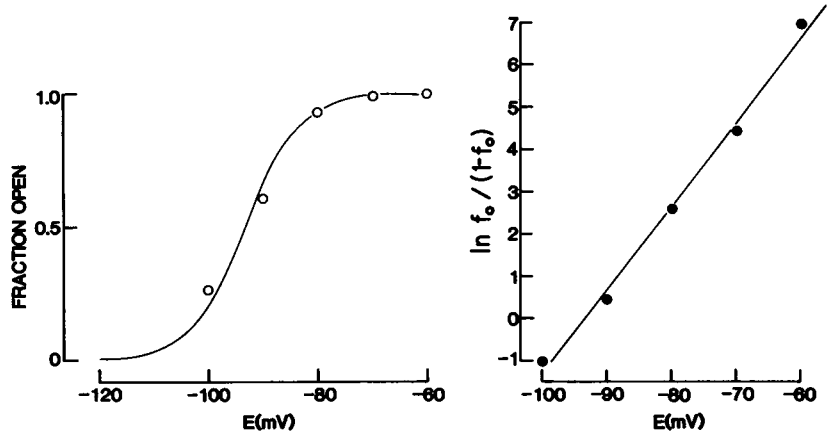

FIGURE 4 Voltage-dependence of channel gating. The fractional open time (f_o) is plotted vs. voltage (*left*) and the same data are plotted in linearized form, $\ln[f_o/(1 - f_o)]$ vs. E, on the right. The linearized data were fit by the assumption that the channel spends 50% of its time open at $E_{0.5} = -93$ mV, and that there is a gating charge of $5e^-$/channel. Further details appear in the text. Data are from the same membrane as those shown in Fig. 3.

The values obtained—an apparent gating charge of 4–6 electronic charges/channel (5.6 ± 1.0, SE, three experiments), and a half-open voltage of $E = -93$ mV (-93 ± 5 mV, SE, three experiments)—are close to those determined from macroscopic current measurements on BTX-activated sodium channels in voltage-clamped neuroblastoma cells (Huang et al., 1982). The key point to be made for the purposes of this paper is that the probability of the channel gates being found open changes significantly with transmembrane potential only over a restricted range of voltages that is more negative (hyperpolarized) than −60 mV.[2]

Saxitoxin Block is Voltage-dependent in a Range Where the Channel Gates Remain Open

The remaining results are based on long records taken in the presence of STX from a membrane containing seven sodium channels. It can be seen from Fig. 5 that all channels are blocked almost all of the time by 320 nM STX at −60 mV, whereas, at +58 mV, up to three, and occasionally four, of the seven channels in the membrane were open simultaneously. At these voltages, channels remained open >98% of the time in the absence of STX (see control record, Figs. 1 and 3). Block by STX thus appears to be voltage-dependent. We have observed, without exception, qualitatively similar voltage dependence in each of six experiments.

To examine the voltage dependence of the steady-state fractional block quantitatively, we determined, from extended recordings, the mean current, corrected for the conductance of the bilayer with no channel conducting. The fraction blocked, f_b, was then estimated as

$$f_b = 1 - f_o \tag{7}$$

where f_o is the fractional open time defined by Eq. 1. Plots of $1/f_b$ vs. $1/[STX]$ were then used to estimate the apparent dissociation constants, at these voltages, for the

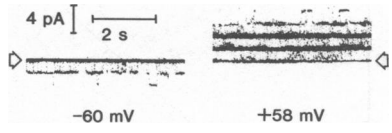

FIGURE 5 Voltage dependence of current fluctuations due to blocking and unblocking of the sodium channels by STX. Current traces from a membrane containing seven sodium channels, with 320 nM STX on the *cis* side, are shown. A downward deflection in the current record represents an inward (*cis* to *trans*) current across the bilayer. At −60 mV, 16 traces are superimposed; at +58 mV, 24 traces are superimposed. The records were filtered (lowpass) at 20 Hz on playback from FM tape. The common level, indicated by the arrows, corresponds to zero current, and the current increment between adjacent levels is ~1.8 pA. Notice that there were never more than two channels simultaneously open at −60 mV, and never more than four open at +58 mV, even though control records showed current corresponding to seven channels. The voltage dependence seen here must be due to a voltage dependence of STX block since the channels remain open essentially all of the time at these voltages in the absence of STX (see control trace, Fig. 1).

[2]Although we have no direct knowledge of either the resting potential in the cells from which our channels originated, or the normal conductance-voltage relation for these channels in the absence of BTX, it seems likely that, as in cellular preparations, BTX positions the conductance-voltage curve at more hyperpolarized potentials than normal. Our ionic conditions, which were chosen to facilitate channel incorporation and to provide a reasonable signal-to-noise ratio, include higher than physiological monovalent ion concentrations and internal (*trans*) calcium, and lower than physiological external (*cis*) calcium. Millimolar concentrations of internal calcium had little or no effect on sodium conductance-voltage relations in squid axon (Begenisich and Lynch, 1975). Lowered external calcium generally shifts conductance-voltage relations in the hyperpolarizing direction (Frankenhauser and Hodgkin, 1957; Hille et al., 1975), but the magnitude of this shift would be reduced by the high monovalent ion concentration (Hille et al., 1975). These data suggest that our ionic conditions might produce a negative shift in the f_o vs. E relation, on the order of 10 mV.

STX block from the equation

$$1/f_b(E) = 1 + K_d(E)/[\text{STX}]. \quad (8)$$

At $E = -60$ mV, we had sufficient data to estimate f_b at only two STX concentrations. In that case we made two discrete estimates of $K_d(-60)$ by substituting $f_b(-60)$ and [STX] directly into Eq. 8 and solving for $K_d(-60\text{ mV})$ (Fig. 9, ○).

As indicated by the increasing slopes of the reciprocal plots in Fig. 6, $K_d(E)$ increases with increasing voltage, a quantitative expression of the observation noted earlier that the degree of block was reduced at positive voltages (Fig. 5). The actual dependence of K_d on E is shown in Fig. 9, where we compare these estimates from steady-state measurements (open symbols) with those from independent kinetic determinations (●).

Voltage Dependence of the Apparent Binding and Dissociation Rates

A marked decrease in the dwell time for the single-channel open level, produced by changing the voltage from +58 mV to −60 mV, is clearly apparent in Fig. 7. This indicates an increase in the binding rate for STX over this range. In Fig. 8, we show the rate constants for binding (blocking), β, and dissociation, λ, calculated from Eqs. 3 and 4, plotted semilogarithmically against voltage. The voltage dependence shown by the two rates is complementary, β increasing and λ decreasing as E is made more negative. The regression lines for the semilogarithmic plots in Fig. 8 suggest that in each of the binding and dissociation steps a single, elementary charge responds to ~30% of the transmembrane voltage or, equivalently, two charges sense 15%. Negative voltages, which attract the cationic toxin toward the inside of the membrane, speed toxin binding and slow

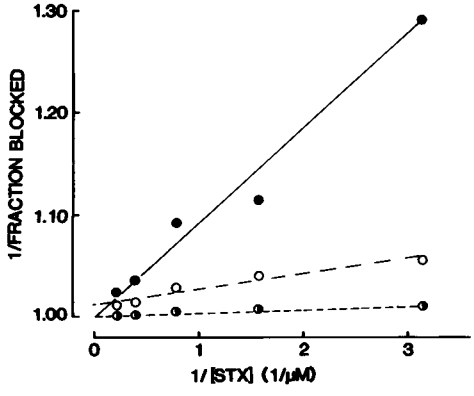

FIGURE 6 Reciprocal plots of dose-response data for STX block at three different voltages. The reciprocal of the fractional block of the sodium channel current is plotted vs. the reciprocal of the STX concentration, with linear regression lines ($r > 0.90$ in each case) for each set of data. Data are from the same membrane as that in Fig. 5. Points shown are for $E = +58$ mV (●), +30 mV (○) and for −30 mV (half-filled circles). Apparent dissociation constants derived from these data are plotted as a function of voltage in Fig. 9.

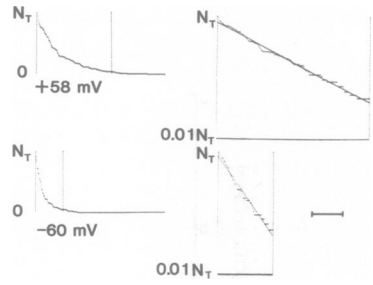

FIGURE 7 Voltage dependence of open-state dwell times. Cumulative dwell time distributions are shown, for $E = +58$ mV and −60 mV for the one-channel–open conductance level, from the same membrane as for Fig. 5. The abscissa represents time, and the ordinate represents the number of events that lasted at least as long as the time, t. *Left*, complete distributions using a linear scale on the ordinate; *right*, semilogarithmic plots of the same data. N_T, the total number of events observed at each potential, was 58 at +58 mV and 75 at −60 mV. The straight lines through the data are linear regression lines through the log-transformed data. In the left-hand parts of the figure, the vertical cursors indicate the segment of the distribution that was used for the fits shown on the right. Notice the marked decrease in the open (unblocked) state dwell times when the voltage is changed from +58 mV to −60 mV. Data were collected in the presence of 320 nM STX. The time scale bar represents 1 s (*left* frame) and 0.5 s (*right* frame).

its dissociation. Quantitatively, for the data in Fig. 8, this information is expressed in the following relations:

$$\lambda_E = \lambda_0 \exp(0.28\, FE/RT) \quad (9)$$

$$\beta_E = \beta_0 \exp(-0.35\, FE/RT) \quad (10)$$

where $\lambda_0 = 0.095\ s^{-1}$ and $\beta_0 = 0.935 \times 10^7\ M^{-1}\ s^{-1}$. The value obtained here for β at $E = 58$ mV ($0.38 \times 10^7\ M^{-1}\ s^{-1}$) may be directly compared with the slope (1.1×10^6

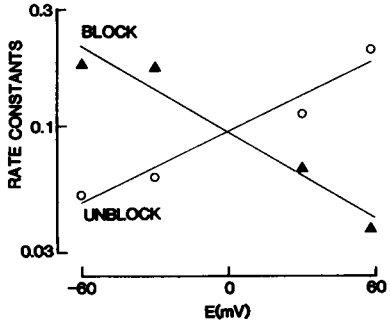

FIGURE 8 Dependence on voltage of the apparent binding and dissociation rate constants for STX block of the channels. The rate constants were derived from the reciprocal dwell times under the assumption that each of the seven channels in the membrane could be independently blocked by STX. The lines shown are linear least-square fits to the logarithm of the rate constants (correlation coefficients, $r = 0.98$ in each case). Notice that the blocking and unblocking rate constants show a voltage dependence of approximately equal steepness but of opposite sign, such that hyperpolarization speeds blocking and slows unblocking. Units on the ordinate are s^{-1} for unblock and $10^8\ M^{-1}\ s^{-1}$ for block. Further details of the analysis are in the text. The rate constants were determined from dwell times measured in the presence of 320 nM STX.

$M^{-1} s^{-1}$) of the plot of reciprocal open dwell times vs. STX concentration in Fig. 2. Part of this discrepancy can be explained by the filtering of the records prior to analysis. As the STX concentration increases, consequently decreasing the open (unblocked) dwell time, the filtering obscures a higher and higher fraction of the open (unblocked) events. At the highest STX concentration used in Fig. 2, up to 10%–20% of the unblocking events would be expected to go unrecognized.

The apparent dissociation constant $K_d(E) = \lambda_E/\beta_E$ may be calculated from these data, and the derived estimates agree closely with those obtained from the steady-state block measurements mentioned earlier (compare open and filled symbols in Fig. 9). In the context of a model in which STX must enter the membrane field (cf. Woodhull, 1973), $K_d(E) = \exp(z\,\delta\,FE/RT)$, where z is the valence ($z = 2$ for STX) and δ is the fraction of the transmembrane voltage sensed by the toxin. When $K_d(E)$, calculated from the blocking and unblocking rate constants, was plotted against membrane potential, we determined a value for $z\delta$ of 0.63 ($\delta = 0.3$), suggesting that the divalent toxin senses ~30% of the transmembrane field when bound in the steady state. When K_d, determined from our steady-state fractional block measurements, was plotted against potential, we obtained a value for δ of ~0.4. Hence, 30–40% of the voltage affects toxin binding, either directly or indirectly.

DISCUSSION

Hille (1968), in describing the block of sodium channel currents in frog node by STX, wrote "... amplitudes of the peak sodium currents were reduced by the same fraction at every voltage, whether the current is inward (negative) or outward." That study, and others (for reviews, see Narahashi, 1974; Ritchie and Rogart, 1977) strongly suggested that one toxin molecule bound to each channel to produce block. Subsequently, kinetic studies (Schwarz et al., 1973) showed that equilibration time constants for TTX—a toxin showing comparable binding affinity, block, and unblock following a step in toxin concentration—were on the order of tens of seconds at concentrations near the dissociation constant. It became clear that measurements of peak sodium current during a depolarizing voltage step from a fixed potential would assay the degree of block for the steady state reached at the holding potential prior to the pulse. Later studies by Almers and Levinson (1975), Ulbricht and Wagner (1975 *a*, *b*), and Cohen et al. (1981) examined the effect on TTX block of steady depolarization preceding a test pulse. In addition, Krueger et al. (1979) examined the effect of potassium-induced depolarization on ^{3}H-STX binding to rat brain membrane vesicles. All of these studies appeared to support the earlier conclusion that block by STX was due to voltage-independent binding with a 1:1 stoichiometry.[3] The question then arises, why should the toxin block appear to be voltage-dependent for BTX-activated sodium channels incorporated into bilayers?

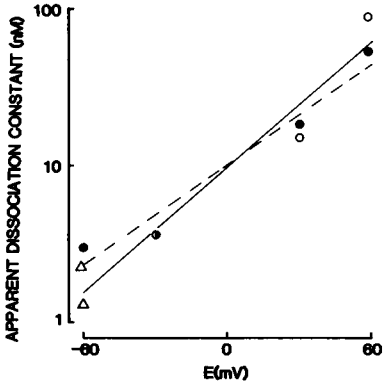

FIGURE 9 Apparent dissociation constants for STX block plotted vs. transmembrane voltage. Two independent determinations are shown. Open symbols (triangles and circles) were obtained from fractional blocked times. The open circles were obtained from the slopes of the regression lines in Fig. 6. Since we only determined the fractional block at two STX concentrations at −60 mV, the two individual points are plotted here (△); the mean of these two points was used, with the open circles, to calculate the linear regression line (—). The solid symbols and the dashed line (---) are the apparent dissociation constants, and the corresponding fit, obtained from the rate constants estimated from the dwell times (see Figs. 7 and 8). The half-filled circle represents the superposition of an open and a solid circle.

There are at least three explanations for this voltage-dependent block by STX. The voltage dependence that we observed may simply have been overlooked because of the length of the equilibration times compared with the usual duration of voltage clamp pulses. The macroscopic relaxation time constant can be calculated from the binding and dissociation rate constants (Fig. 8) using the expression

$$\tau_{rel} = 1/(\lambda + \beta[\mathrm{STX}]). \quad (11)$$

Using the rate constants for $E = 0$ mV, $\tau_{rel} = 5.3$ s when [STX] = 10 nM. Although the long relaxation times for TTX and STX block were not taken into account in some earlier studies, this first possibility can probably be discarded on the basis of the careful studies of Ulbricht and Wagner (1975 *a*, *b*), Almers and Levinson (1975), and Cohen et al. (1981).

The second possibility is that BTX modification causes the toxin block to become voltage-dependent. This possibility is of interest in light of the observation that BTX induces changes in the ion selectivity of sodium channels (see Khodorov, 1978, for a brief review). To date, we have been unable to address this question in our preparation, since with the relatively large bilayers used in these studies we lacked the necessary current-time resolution to study

[3] A report by Baer et al. (1976) that depolarization enhances TTX block of cardiac sodium channels has been challenged on technical grounds (Cohen and Strichartz, 1977; Cohen et al., 1981) and is not supported by more recent voltage clamp studies (Colatsky and Gadsby, 1980; Cohen et al. 1981).

sodium-channel currents in the absence of BTX. However, published reports suggest that both binding of ^{3}H-STX (Krueger et al., 1979) and block of sodium channels by TTX (Mozhayeva et al., 1982) are unaffected by BTX activation.

The third possibility is that one of our experimental conditions either induces or accentuates the voltage dependence. Specifically, we used calcium and sodium at the physiological inner surface of the channels in concentrations about 100-fold higher than those found in intact nerve. Binding of a cation to a site accessible from the inside, within the transmembrane field, might repel the cationic toxin from a binding site at the outer surface, and would thus confer voltage-dependence on the block, with the orientation that we observed. At concentrations far below their dissociation constants, binding of the "competing" ions would be negligible regardless of voltage, hence voltage would have no significant effect on STX binding. This mechanism is related to the "modified competition" proposed by Ulbricht and Wagner (1975 *a*) to account for an apparent voltage dependence of TTX binding seen only at low pH. The third possiblity could be tested by varying the composition of the solution at the inner surface of the channels (the *trans* side). Specifically, if STX binding (which may be intrinsically voltage-independent) can be prevented or reduced by the occupancy of a cation binding site accessible from the inside and within the membrane field (i.e., occupancy of that site would be voltage-dependent), then the apparent voltage dependence of STX block should be reduced by lowering the sodium and/or calcium concentrations on the inside of the membrane. We are now performing experiments to test this possibility.

With regard to the specific molecular basis of the voltage dependence, it will also be of interest to examine the effect of voltage on TTX block of these channels, since TTX is monovalent at physiological pH, while STX is divalent. If these blockers enter the transmembrane electric field to block the channels, one might expect the block by STX to be more strongly influenced by voltage. Nonetheless, the experiments may not necessarily lead to an unambiguous conclusion, as the effect of voltage on the action of divalent cations with two separated charges can show a complex dependence on their size and molecular structure (Miller, 1982). The larger size of STX (the charges are separated by ~3 Å) makes it possible that each of the charged groups would penetrate a different fraction of the transmembrane voltage.

Two observations have been made in other systems, from which it appears that depolarization may be able to reduce the degree of TTX block. In cardiac muscle, a slight reduction, by depolarization, of block of background sodium conductance is shown in Fig. 1 *C* of Colatsky and Gadsby (1980). This parallels our own observations, but the effect is less marked. Although Mozhayeva et al. (1982) were addressing an apparently unrelated phenomenon (viz., an irreversible effect of TTX on gating of BTX-activated sodium channels), their records (Fig. 1 *C*) show a slow, steady increase in current amplitude toward the end of 50–100-ms depolarizing pulses. This could be the result of slowly relaxing, depolarization-induced release of TTX block of the BTX-activated channels.

We thank Carl Phillips for preparing the figures, G. Ay for fabricating the chambers, J. Michael and T. Sparks for assisting with the electronics, and Brian Hall for help installing an updated version of DAOS software. We are expecially indebted to Dr. J. Daly for providing a generous supply of BTX for these studies. We also thank Drs. M. S. Brodwick, M. D. Cahalan, D. C. Eaton, L. Goldman, P. A. Pappone, R. Payne, J. J. Shoukimas, G. Strichartz, and R. W. Tsien for helpful discussions and comments.

This work was supported by National Institutes of Health grants NS-16775 to R. J. French and NS-16285 to B. K. Krueger, by the Bressler Foundation of the University of Maryland School of Medicine, and by contract DAMD-17-C-2188 from the U.S. Army Medical Research and Development Command, both awarded to B. K. Krueger and R. J. French.

Received for publication 2 May 1983 and in final form 11 June 1983.

REFERENCES

Albuquerque, E. X., and J. Daly. 1976. Batrachotoxin, a selective probe for channels modulating sodium conductances in electrogenic membranes. *In* The Specificity and Action of Animal, Bacterial, and Plant Toxins. Receptors and Recognition, Series B. P. Cuatrecasas, editor. Chapman and Hall, London. 1:297–338.

Almers, W,. and S. R. Levinson. 1975. Tetrodotoxin binding to normal and depolarized frog muscle and the conductance of a single sodium channel. *J. Physiol.* (*Lond.*). 247:483–509.

Baer, M., P. M. Best, and H. Reuter. 1976. Voltage-dependent action of tetrodotoxin in mammalian cardiac muscle. *Nature* (*Lond.*). 263:344–345.

Begenisich, T., and C. Lynch. 1975. Effects of internal divalent cations on voltage clamped squid axons. *J. Gen. Physiol.* 63:675–689.

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

Cohen, F. S., M. H. Akabas, and A. Finkelstein. 1982. Osmotic swelling of phospholipid vesicles causes them to fuse with a planar phospholipid bilayer membrane. *Science* (*Wash., DC*). 217:458–460.

Cohen, F. S., J. Zimmerberg, and A. Finkelstein. 1982. Fusion of phospholipid vesicles with planar phospholipid bilayer membranes. II. Incorporation of a vesicular membrane marker into the planar membrane. *J. Gen. Physiol.* 75:251–270.

Cohen, I. S., and G. R. Strichartz. 1977. On the voltage-dependent action of tetrodotoxin. *Biophys. J.* 17:275–279.

Colatsky, T. J., and D. C. Gadsby. 1980. Is tetrodotoxin block of background sodium channels in canine cardiac Purkinje fibers voltage-dependent? *J. Physiol.* (*Lond.*). 206:20P.

Colquhoun, D., and A. G. Hawkes. 1981. On the stochastic properties of single ion channels. *Proc. R. Soc. B. Biol. Sci.* 211:205–235.

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

French, R. J., and R. Horn. 1983. Sodium channel gating: models, mimics and modifiers. *Annu. Rev. Biophys. Bioeng.* 12:319–356.

Hille, B. 1968. Pharmacological modifications of the sodium channels of frog nerve. *J. Gen. Physiol.* 51:199–219.

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

Huang, L. -Y. M., N. Moran, and G. Ehrenstein. 1982. Batrachotoxin modifies the gating kinetics of sodium channels in internally perfused neuroblasoma cells. *Proc. Natl. Acad. Sci. USA.* 79:2082–2085.

Khodorov, B. I. 1978. Chemicals as tools to study nerve fiber sodium channels: effects of batrachotoxin and some local anesthetics. *In* Membrane Transport Processes. D. C. Tosteson, Y. A. Ovchinnikov, and R. Latorre, editors. Raven Press, New York. 2:153–174.

Krueger, B. K., R. W. Ratzlaff, G. R. Strichartz, and M. P. Blaustein. 1979. Saxitoxin binding to synaptosomes, membranes, and solubilized binding sites from rat brain. *J. Membr. Biol.* 50:287–310.

Krueger, B. K., J. F. Worley, and R. J. French. 1983. Single sodium channels from rat brain incorporated into planar lipid bilayer membranes. *Nature (Lond.).* 303:172–175.

Labarca, P., R. Coronado, and C. Miller. 1980. Thermodynamic and kinetic studies of the gating behavior of a K+-selective channel from the sarcoplasmic reticulum membrane. *J. Gen. Physiol.* 76:397–424.

Miller, C. 1978. Voltage-gated cation conductance channel from fragmented sarcoplasmic reticulum: steady-state electrical properties. *J. Membr. Biol.* 40:1–23.

Mozhayeva, G. N., A. P. Naumov, and B. I. Khodorov. 1982. Tetrodotoxin changes the activation kinetics of batrachotoxin-modified sodium channels. *Gen. Physiol. Biophys.* 1:221–223.

Miller, C. 1982. Bis-quaternary ammonium blockers as structural probes of the sarcoplasmic reticulum K channel. *J. Gen. Physiol.* 79:869–891.

Mueller, P., D. O. Rudin, H. T. Tien, and W. C. Wescott. 1963. Methods for the formation of single bimolecular lipid membranes in aqueous solution. *J. Phys. Chem.* 67:534–535.

Narahashi, T. 1974. Chemicals as tools in the study of excitable membranes. *Physiol. Rev.* 54:813–866.

Quandt, F. N., and T. Narahashi. 1982. Modification of single Na^+ channels by batrachotoxin. *Proc. Natl. Acad. Sci. USA.* 79:6732–6736.

Ritchie, J. M., and R. B. Rogart. 1977. The binding of saxitoxin and tetrodotoxin to excitable tissue. *Rev. Physiol. Biochem. Pharmacol.* 79:2–50.

Schwarz, J. R., W. Ulbricht, and H. -H. Wagner. 1973. The rate of action of tetrodotoxin on myelinated nerve fibres of *Xenopus laevis* and *Rana esculenta. J. Physiol. (Lond.).* 233:167–194.

Ulbricht, W., and H. -H. Wagner. 1975*a*. The influence of pH on equilibrium effects of tetrodotoxin on myelinated nerve fibers of *Rana esculenta. J. Physiol. (Lond.).* 252:159–184.

Ulbricht, W., and H. -H. Wagner. 1975*b*. The influence of pH on the rate of tetrodotoxin action on myelinated nerve fibers. *J. Physiol. (Lond.).* 252:185–202.

Woodhull, A. M. 1973. Ionic blockage of sodium channels in nerve. *J. Gen. Physiol.* 61:687–708

DISCUSSION

Session Chairman: Alan Finkelstein *Scribes*: Lawrence B. Weiss, Juli Lai Weiss, and Charles L. Bowman

PAPPONE: In your paper you give two possible explanations for the voltage dependence of STX block of BTX-modified Na^+ channels. It could be an effect of the BTX modification or of the unphysiologic Na^+ concentration on the *trans* side. Do you have any data which address this question?

FRENCH: These may be two distinct issues. Firstly, is BTX modification required for the voltage dependence? Secondly, when voltage dependence occurs, is it due to an interaction between STX and some "internal" ion? At this point, we have not done experiments at wide enough bandwidths to study the channels in the absence of BTX and thus resolve the question of primary BTX effect. Krueger et al. have addressed this in K^+-depolarized synaptosomes and found no effect of BTX on STX binding. This question is raised for our system because the Na^+ concentration on the *trans* side was orders of magnitude higher than physiologic concentration. Therefore, the possibility is raised that Na^+ may displace BTX. We have no direct evidence for this.

PAPPONE: Because you have interpreted the voltage dependence of block by STX to mean that STX has moved into the channel and because STX and TTX have different numbers of charges, have you compared the voltage dependence of these two toxins?

FRENCH: Yes. We have some preliminary results and have found that TTX exhibits the same voltage dependence as STX.

FINKELSTEIN: Is it correct that for both STX and TTX there is an *e*-fold change in block for 40 mV?

FRENCH: Yes.

RUBINSON: I would argue that it is something other then the movement of the next charge on the toxin in the membrane field that is causing the effect.

FRENCH: I would agree.

FINKELSTEIN: Could you clarify your point about the experimental Na^+ concentrations?

FRENCH: We used symmetric 0.5 M NaCl in our experiments.

ANDERSEN: In collaboration with Bill Green and Larry Weiss, I have done similar experiments with dog brain synaptosomes. Barring any major species difference, we have encountered the same voltage dependence for TTX binding to BTX-modified Na^+ channels. This voltage dependence is comparable with that reported here and is independent of Na^+ concentration. We have also found the K_D for TTX block at a given potential varies with Na^+ concentration. Between 50 mM and 2.5 M NaCl there is no change in the voltage dependence of TTX block and there appears to be a competitive interaction between Na^+ concentration and TTX block. This raises the question of whether or not TTX and STX bind in the permeation pathway. Further, although the block induced by STX and TTX are voltage dependent, we don't know if the binding of these toxins is voltage dependent or if the block represents an independent process. Is there any evidence whether these neurotoxins bind in the permeation pathway or whether they bind at an allosteric site?

FRENCH: There is no direct evidence. The idea that STX and TTX may act as a plug is suggested by the fact that these toxins contain guanidinium groups, and that guanidinium can carry current through the Na^+ channel.

YEH: The results of our (Tanguy, Narahashi, and Yeh, unpublished) experiment with BTX on Na^+ channels in squid axon membranes support the idea that BTX modification causes the toxin (STX) block to become voltage-dependent. As had been reported in many other preparations, BTX has two major effects on the gating kinetics in squid axon; it opens Na^+ channels at very negative potentials and it removes Na^+ inactivation. In addition, BTX-modified Na^+ channels have different pharmacological profiles. For example, in the presence of 10 nM STX, the Na^+ current at -80 mV was suppressed to ~50% of the control, whereas at $+20$ mV, the

suppression was <20%. Thus the blocking action of STX in BTX-modified channels is voltage dependent.

Furthermore, this voltage-dependent block occurs very rapidly, as reflected in the lack of time dependence of the unblocking rates. This is in marked contrast to the effect of STX on normal Na channels. As French pointed out, STX block of normal Na^+ channels is not voltage dependent and the unblocking rate is rather slow.

MOCZYDLOWSKI: In Chris Miller's lab, we have been studying Na^+ channels from rat muscle in bilayers and our data confirm that of Olaf Andersen and Robert French with respect to the voltage dependence of toxin binding. In collaboration with Gary Strichartz and Sherwood Hall, we have studied six different derivatives of STX in which the net charge is modified by sulfate groups. The various toxins bear a net charge from 0 to +2. All these toxins exhibit the same voltage dependence. Our findings on Na^+ competition reveal only an effect on the association rate with no effect on the off rate constant in the range of 40–600 mM Na^+. In the simple competitive model, this suggests that the binding constant for Na^+ is voltage independent while toxin binding to this site is voltage dependent.

FRENCH: Those observations argue against the voltage dependence being due to Na^+ from the inner side because this would probably modify the off rate.

ANDERSEN: We find no change in the off rate constant from 50 mM to 2.5 M Na^+, making it unlikely that there is an effect of Na^+ from the inner side. There is, however, a flaw in this argument. If there is a 1.0 mM K_D for Na^+ to an inner binding site, we may really be looking at a Na^+–TTX interaction in a doubly occupied channel.

HALL: Because your experiments are done in decane-containing membranes which exhibit electrostrictive thinning, can you be sure that this deformation is not the origin of your voltage dependence?

MOCZYDLOWSKI: We have done experiments in folded membranes without decane, and our results are the same.

BENNETT: If the toxin site is first in the permeation pathway and then in the blocked state, the entire potential might drop across the single guanidinium group, independent of the rest of the molecule.

FRENCH: Such a potential profile offers a possible way of explaining the steep voltage dependence of action of the bulky toxin molecules, but it also raises two questions. Why don't we see a voltage dependence corresponding to the whole voltage drop? And how would this picture apply to the more complex derivatives that Ed Moczydlowski and Chris Miller have been studying?

MOCZYDLOWSKI: What you're proposing is that only one guanidinium is entering the field, while the second guanidinium would be completely excluded even though the distance between these two groups is 3–4 Å for the saxitoxin molecule. This explanation seems unlikely.

FINKELSTEIN: To rephrase this important question: in the simple blocking models it has been assumed that the electric field is the same in the blocked and unblocked states, but this model may not be correct. It may be more correct to ask, what is the voltage profile that the blocking ion sees when the site is unblocked? And what does it see after the site is blocked?

BARCHI: Is the evidence you have presented consistent with a very small voltage-dependent change in tertiary or quarternary structure in the binding site itself?

FRENCH: Yes.

ANDERSEN: I would like to point out another very interesting finding, by Almers and Levinson (1975. *J. Physiol.* (*Lond.*). 247:483), which is usually cited as evidence for voltage-independent block but which actually supports voltage-dependent block. In a muscle preparation in normal NaCl they determined a K_D for TTX binding. These muscles were then K^+ depolarized in a Na^+-free solution and the K_D was unchanged. However, other investigators (Reed and Raftery. 1976. *Biochemistry.* 15:944; Barchi and Weigele. 1979. *J. Physiol.* (*Lond.*). 295:383) as well as ourselves, have demonstrated Na^+ competition for TTX binding; thus, one would expect an increase in K_D of three–fivefold. If one now assumes by going to the Na-free solution a 50–70 mV change in membrane potential, which should decrease the K_D three–fivefold, then one would see no significant change in K_D. This was their observation.

KRUEGER: It is interesting that our results, which demonstrate voltage-dependent TTX and STX block, were obtained with BTX-activated sodium channels that were open in the steady state. This is in contrast to other work in which the membrane potential was held for long times at either hyperpolarized or depolarized potentials at which the channels were either closed or inactivated. It is of interest that Colatsky and Gadsby (1980. *J. Physiol.* (*Lond.*). 306:20P) reported using a heart preparation to study the steady-state block of open Na^+ channels under conditions where the channels were open but not inactivated. They found a small but significant shift in the steady-state binding constant for TTX in the same direction as we report here.

ADELMAN: Once TTX or STX block has been established, can you relieve it with a change in voltage and is it time dependent?

FRENCH: This has not been done quantitatively, but on changing the membrane potential one can see the relaxation to a new level of block within seconds.