

Batrachotoxin-activated Na⁺ Channels in Planar Lipid Bilayers

Competition of Tetrodotoxin Block by Na⁺

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ABSTRACT Single Na⁺ channels from rat skeletal muscle plasma membrane vesicles were inserted into planar lipid bilayers formed from neutral phospholipids and were observed in the presence of batrachotoxin. The batrachotoxin-modified channel activates in the voltage range -120 to -80 mV and remains open almost all the time at voltages positive to -60 mV. Low levels of tetrodotoxin (TTX) induce slow fluctuations of channel current, which represent the binding and dissociation of single TTX molecules to single channels. The rates of association and dissociation of TTX are both voltage dependent, and the association rate is competitively inhibited by Na⁺. This inhibition is observed only when Na⁺ is increased on the TTX binding side of the channel. The results suggest that the TTX receptor site is located at the channel's outer mouth, and that the Na⁺ competition site is not located deeply within the channel's conduction pathway.

INTRODUCTION

The primary event in the propagated action potential of skeletal muscle and nerve is a transient inward current of Na ions mediated by a voltage-dependent Na⁺ channel. The operation of this channel has been under study for over three decades in excitable cell membranes, and recent investigations have entered the domain of biochemistry with the purification of the channel protein (for a review, see Catterall, 1984). Because of the central importance of the Na⁺ channel in membrane excitability, most higher organisms are vulnerable to a variety of natural toxins directed against it (Catterall, 1980). In this report, two such toxins are used to provide a functional characterization of Na⁺ channels at an intermediate level of biochemical simplicity: that of a planar phospholipid bilayer membrane into which single channels are inserted from native plasma membrane vesicles.

To examine the channel's behavior under steady state conditions over a wide

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voltage range, we used batrachotoxin (BTX) to prevent inactivation of channels incorporated into planar bilayers, a strategy first employed by Krueger et al. (1983). Our results show that the BTX-activated channel is open almost all the time at voltages more positive than -60 mV, and that unitary blocking events induced by tetrodotoxin (TTX) represent the binding of individual TTX molecules to the channel.

The simplest mechanism of channel blockade is direct occlusion of the aqueous pore by the blocking molecule, as has been proposed for the action of guanidinium toxins like TTX (Kao and Nishiyama, 1965; Hille, 1975*a*). An early detailed hypothesis for TTX blockade placed the location of the toxin guanidinium group in an intimate region of the Na^+ conduction pathway known as the "selectivity filter," where ion-pair formation between the guanidinium group and a carboxylate residue of the channel protein was presumed to occur (Hille, 1975*a*). In contrast, a more recent hypothesis placed the toxin binding site completely outside of the ion conduction pore on the surface of the channel mouth (Kao and Walker, 1982). Thus, there are mechanistic distinctions between "deep" and "shallow" locations of blocking sites for ion channels. In this study, we investigate the location of the guanidinium toxin receptor in relation to the ion permeation pathway by examining, at the single channel level, the characteristics of relief of TTX block by a permeant ion, Na^+ . The results here and in the companion study (Moczydlowski et al., 1984) argue against a direct correlation between the sites involved in Na^+ permeation and those involved in Na^+ competition with toxin blockade. Our overall conclusion is that the site of guanidinium-toxin binding resides at a shallow location on the channel protein.

MATERIALS AND METHODS

Chemicals and Membrane Preparation

TTX, obtained from Calbiochem-Behring (San Diego, CA), was stored in stock solutions containing 1 mM citrate, pH 5. Phospholipids used were phosphatidylcholine (PC) from egg yolks, and phosphatidylethanolamine (PE) from bovine brain, purchased from Avanti Polar Lipids (Birmingham, AL). BTX was the generous gift of Dr. John Daly, Laboratory of Bio-organic Chemistry, National Institutes of Health, Bethesda, MD.

Plasma membrane vesicles were prepared from rat skeletal muscle, as described (Moczydlowski and Latorre, 1983*a*), except that the Ca^{2+} loading step was usually omitted. Light vesicles banding on a cushion of 34% sucrose (wt/vol) were washed and stored at ~ 10 mg/ml in 300 mM sucrose at -70°C .

Planar Bilayers and Channel Insertion

Planar bilayers were cast on 100–200- μm holes in polystyrene partitions from decane solutions containing PE (16 mg/ml) and PC (4 mg/ml), and current was monitored at constant holding voltages, using low-noise electronics, as described (Hanke and Miller, 1983). Standard aqueous buffer solution was 200 mM NaCl, 0.1 mM EDTA, 0.2 μM BTX, 10 mM MOPS-NaOH, pH 7.4; in some experiments, the NaCl concentration was varied over the range 3–600 mM. Unless otherwise noted, both sides of the bilayer contained aqueous solutions of identical composition.

Insertion of Na^+ channels could be observed in the presence of BTX, essentially as described by Krueger et al. (1983). With the preparation used here, we consistently

obtained membranes containing only one or two channels, which remained stable for up to several hours. Incorporation was induced by adding plasma membrane vesicles (20–40 $\mu\text{g}/\text{ml}$) to the *cis* side of the bilayer, with a holding voltage of 30–50 mV. Channel insertion usually occurred spontaneously within 30 min and was detected as an abrupt increase in conductance of ~ 20 pS. Channels nearly always inserted with the TTX-receptor side facing the *cis* aqueous chamber. For consistency, all voltages are defined in accordance with the cellular convention; the *cis* side of the bilayer is defined as zero voltage, so that positive or depolarizing potentials favor the opening of Na⁺ channels.

Similarly, we incorporated Na⁺ channels into virtually solvent-free bilayers formed by folding two lipid monolayers at the air-water interface over a hole in a Teflon partition (Montal and Mueller, 1972), as described previously (Hanke and Miller, 1983). All experiments were performed at $24 \pm 1^\circ\text{C}$ in a thermostatted chamber.

For planar bilayers of this size (100–200 pF capacitance), single Na⁺ channels could be recorded at 500 Hz resolution. However, for analysis of slow TTX blocking events, 100-Hz filtering was sufficient. Records were stored on FM tape and later analyzed by computer or by hand.

Analysis of TTX Block

In order to avoid contamination of the slow TTX blocking events with channel gating events, we chose a cutoff time, α , to distinguish the two types of events. Any event longer than α was considered a TTX block, while any event shorter than this was not counted in the construction of dwell-time histograms. Since the blocked-time distributions were exponential, explicit corrections could be made for overestimation of the true mean due to the cutoff limit. The observed mean, τ'_b , of an exponential distribution truncated by a lower limit, α , is related to the true mean, τ_b , by (Neher and Steinbach, 1978):

$$\tau_b = \tau'_b - \alpha. \quad (1)$$

Similarly, the observed unblocked time, τ'_u , is related to its true mean, τ_u , by (Sachs et al., 1982):

$$\tau_u = \tau'_u \exp(-\alpha/\tau_b). \quad (2)$$

These relations were used to correct the observed dwell times for the cutoff limit. For all experiments here, we chose a cutoff time of 400 ms. Given the large differences in the blocking and gating times, this choice led to a fidelity of identifying blocking events of >98%, and to corrections for mean times of <10%.

Association and dissociation rate constants were calculated as the inverse mean unblocked and blocked times, respectively, using data from membranes containing only one Na⁺ channel. A few analyses were performed on membranes containing two channels. In the latter case, rate constants of blocking and unblocking were extracted from the multiple-level records using binomial statistics (Labarca et al., 1980; Hanke and Miller, 1983).

RESULTS

Characteristics of BTX-activated Na⁺ Channels

Under physiological conditions, the Na⁺ channel may be activated by applying a depolarizing voltage. However, this activation is only transient; after a millisecond, the channel spontaneously inactivates. BTX has been shown to eliminate the inactivation process as well as to shift the voltage dependence of the activation process to more negative potentials (Huang et al., 1982; Dubois et al., 1983).

Thus, with BTX-modified channels, blocking reactions can be studied at equilibrium, over a range of voltages that normally silence the channel. We first present identifying features and pertinent gating characteristics of the BTX-modified Na^+ channel and then describe experiments that explore the mechanism of inhibition of this channel by TTX.

Fig. 1 illustrates typical recordings obtained in a bilayer containing a single BTX-activated Na^+ channel. In this and all other figures, records are displayed so that channel opening corresponds to an upward deflection. These records represent true steady state conditions: we have never observed any inactivation of these channels following voltage pulses. Such channels can easily be observed opening and closing for hours at depolarizing voltages that would, in the absence of BTX, lead to inactivation of the channel in milliseconds. The figure shows that applied voltage controls the characteristics of the channel. At voltages more positive than -60 mV, the channel is nearly always in its open state, as typified by the trace at $+80$ mV. As voltage is made more negative than -60 mV, brief closing events become increasingly prominent, until the channel is nearly always closed at voltages negative to -120 mV. This behavior is similar to that first observed by French and co-workers (1984) for the rat brain Na^+ channel in planar bilayers.

The probability of observing the open state, P_o , is shown in Fig. 2 as a function of applied voltage. A steep voltage dependence, qualitatively reminiscent of the normal Na^+ channel activation curve, is evident. The equilibrium constant for opening changes e-fold per 6.7 mV, and the opening probability is half-maximal at -94 mV. This "activation curve" agrees with previous results for brain channels

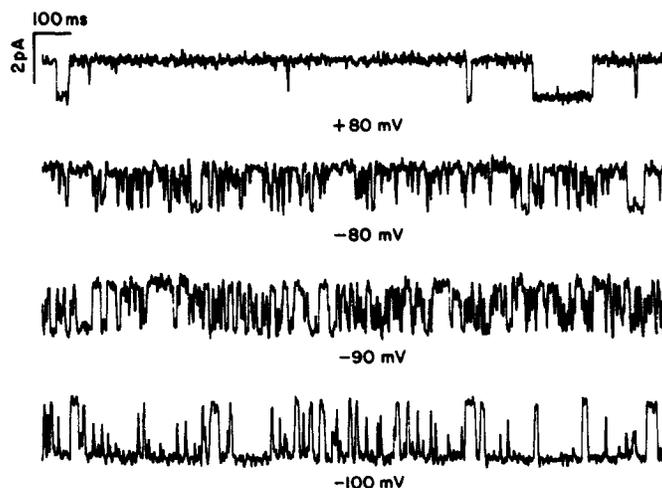


FIGURE 1. A single Na^+ channel in a decane-containing bilayer. Representative records from the same bilayer are shown at the indicated holding voltages. Closing transitions are downward in all records. The bilayer was cast from a 20 mg/ml solution of lipids in decane. Aqueous phase was 200 mM NaCl, 25 mM MOPS-NaOH, 0.1 mM EDTA, pH 7.4. The measured probabilities of residence in the open state, averaged over 20 s, were (top to bottom): 0.93, 0.87, 0.57, 0.22. Data were filtered at 400 Hz.

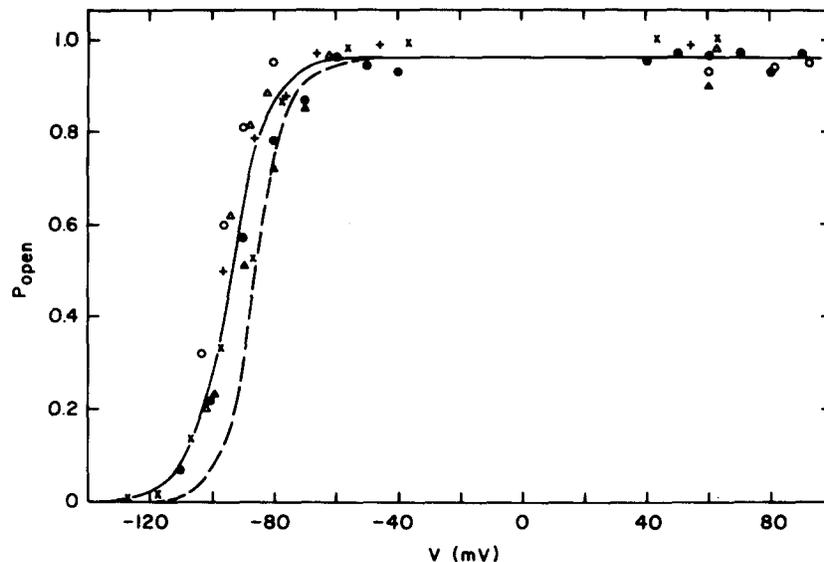


FIGURE 2. Voltage dependence of BTX-modified Na⁺ channels in planar bilayers. The different symbols correspond to data points from six different single channel bilayers. The bilayers were formed from a PE/PC lipid mixture using either the Mueller-Rudin (circles, triangles) or Montal-Mueller (crosses, x's) technique. The buffer was MOPS, pH 7.4, with NaOH and 200 mM NaCl or Na⁺-acetate. The time-averaged probability of the open state, P_o , was measured as in Methods. Data from each bilayer were fit to the equation: $\ln[(P_o/P_{\max} - P_o)] = A(V - V_o)$. The solid line was drawn using the average values: $P_{\max} = 0.96$, $A = 0.15 \text{ mV}^{-1}$, $V_o = -94 \text{ mV}$. The dashed curve is the fit of Dubois et al. (1983) for the open state probability of BTX-activated Na⁺ channels in frog node of Ranvier.

in planar bilayers (French et al., 1984), and with results in neuroblastoma cells (Huang et al., 1982), which showed that BTX shifts the normal activation curve by about -50 mV along the voltage axis. Moreover, these data in planar bilayers agree with the activation curve for BTX-modified Na⁺ channels in frog node (Dubois et al., 1983), presented as a dashed curve in Fig. 2 for comparison.

In addition to voltage-dependent activation, the channel under study here has a unit conductance and selectivity properties that confirm its identity as the action potential Na⁺ channel. We find that the current-voltage relation of the open channel is linear over the range -100 to 100 mV , with a single channel conductance of 20 pS at 200 mM NaCl (Fig. 3). From measurements of reversal potentials under mixed-ion conditions (Fig. 3), we find the selectivity sequence expected for Na⁺ channels: $\text{Li}^+ \sim \text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+ \gg \text{Cl}^-$ (Table I). As discussed below, these results are in agreement with recent measurements of mammalian Na⁺ channels using patch recording (Patlak and Horn, 1982) and ion flux measurements (Huang et al., 1979).

Despite these similarities, we were concerned that the decane content of the planar bilayers might affect the channel, as suggested by the documented effects of alkanes on squid axon Na⁺ currents (Haydon et al., 1977). To address this

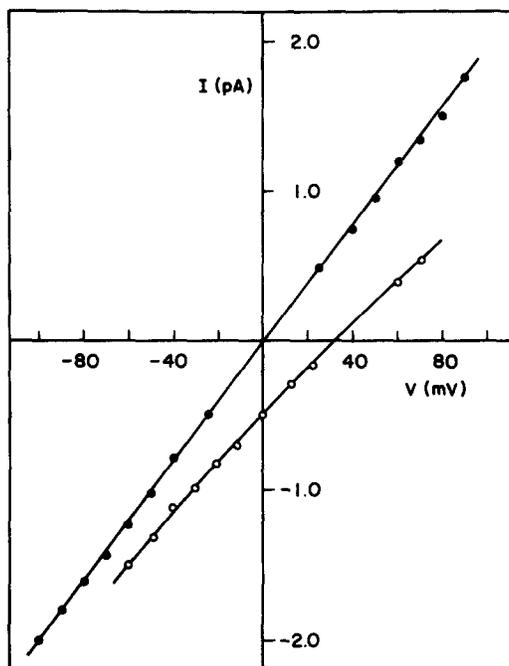


FIGURE 3. Open channel current-voltage relation. Open channel current-voltage curves were measured in bilayers containing single Na^+ channels under different ionic conditions. Filled points: symmetrical NaCl, 250 mM. Open points: 250 mM NaCl on *cis* side, 200 mM KCl + 50 mM NaCl on *trans* side. The reversal potential in the mixed salt experiment was 32 mV.

question, we studied the behavior of Na^+ channels in practically solvent-free bilayers formed from apposed surface monolayers of the same phospholipids used in the experiments above. Although it was much more difficult to induce the insertion of channels into folded bilayers, we could collect enough data to show that the gating behavior in the folded membranes is indistinguishable from

TABLE I
Ion Selectivity of BTX-modified Na^+ Channels

Ion	$P_{\text{ion}}/P_{\text{Na}}$
Li^+	1.0, 0.42*
K^+	0.07
Rb^+	≤ 0.05
Cs^+	≤ 0.01

Data are permeability ratios for various monovalent cations calculated from measured reversal potentials. Reversal potentials were obtained from single channel currents measured under the following conditions: 0.25 M NaCl (*trans*), 0.05 M NaCl + 0.2 M XCl (*cis*), where XCl is the chloride salt of the indicated ion. Since the actual reversal current was too small to measure for Rb^+ and Cs^+ , the reported value is an upper limit based on an extrapolation to the voltage axis.

* This value is the ratio of the measured conductance of the channel in symmetrical solutions of 0.2 M LiCl (8.4 pS) and 0.2 M NaCl (20 pS).

that in the decane-laden membranes (Figs. 1, 2, and 4). In addition, the unit conductance and TTX blocking kinetics were similar in the two types of bilayers (data not shown).

One unexpected gating phenomenon of this channel, both in decane-containing and solvent-free bilayers, is that in the range -100 to -70 mV, the channel's behavior is not entirely stationary. In this "gating" range of holding voltages, we occasionally observed sudden shifts in gating activity, as though the activation curve suddenly (and randomly) shifted by 15–20 mV along the voltage axis (Fig. 5). This phenomenon has been observed previously with glutamate-activated channels of insect muscle (Gration et al., 1981), and in Ca^{2+} -activated K^+ channels from rat muscle inserted in planar bilayers (Moczydlowski and Latorre, 1983*b*). These shifts are infrequent enough so that they do not compromise our data analysis.

At present, our 500-Hz time resolution is limited by high-frequency noise due to the large bilayer capacitance, and thus many of the transitions in the gating region are incompletely resolved (Figs. 1 and 4). However, we have carried out a dwell-time analysis of fluctuations at voltages positive to -60 mV, since most of the closing events in this region can be resolved and are pertinent to subsequent analysis of blockade. At these voltages, the channel spends most of its time in the open state, with occasional brief closings, as in the record shown in Fig. 6 at $+70$ mV. A closer examination of these closures at an expanded time scale (i.e., Fig. 1, top) suggests qualitatively that there are two types of closing events: a class of well-resolved closings lasting tens of milliseconds, and a poorly resolved, shorter-lived class. This impression is quantitatively confirmed in Fig. 6, which shows

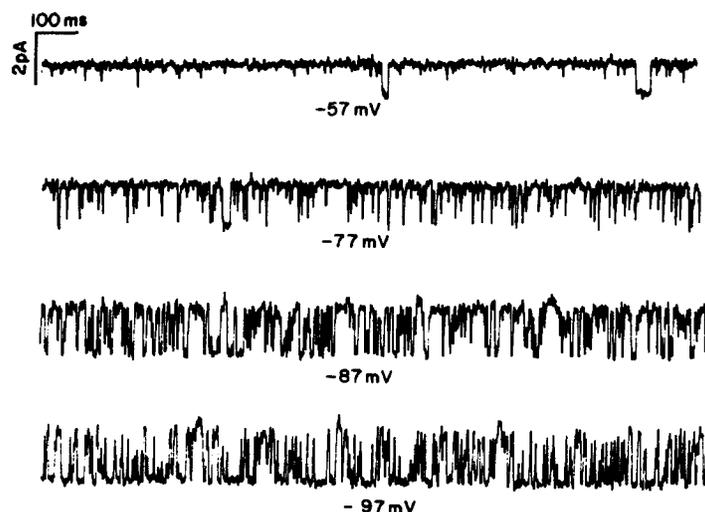


FIGURE 4. Single Na^+ channels in solvent-free bilayers. Conditions were as in Fig. 1, except that the bilayer was formed from solvent-free apposed monolayers of lipids. The solution on both sides of the bilayer was 200 mM Na^+ -acetate, 10 mM Hepes-NaOH, 0.1 mM EDTA, pH 7.4. The measured probabilities of residence in the open state were (top to bottom): 0.98, 0.88, 0.53, 0.33.

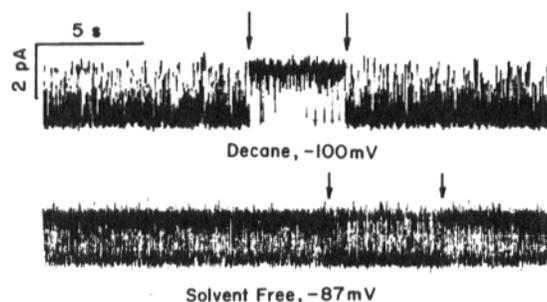


FIGURE 5. Examples of nonstationary gating kinetics of single Na^+ channels. (Top) Decane-containing bilayer, as in Fig. 1, at -100 mV holding potential (100-Hz filter). (Bottom) Solvent-free bilayer, as in Fig. 4, at -87 mV holding potential (400-Hz filter). (Top) The probability of being open briefly shifted from 0.2 to 0.9; (bottom) a similar brief shift from 0.7 to 0.3 occurred. These brief segments of shifted gating are marked by arrows.

that the closed dwell times can be fit well by a double-exponential probability distribution. The open state, on the other hand, follows a single-exponential distribution.

A striking result is that in the range -60 to $+80$ mV, there is no discernible voltage dependence of the single channel gating kinetics. The time constant for the slow component of the closed time is 60 ms, while that of the open time is ~ 800 ms (means of 12 different membranes). Neither of these parameters varies with applied voltage, although the mean open time varies from channel to channel. These closing transitions are probably unrelated to the voltage-dependent gating seen in the range -120 to -80 mV, but may indicate another channel closing process whose significance we do not understand. They may be related in some way to the inactivated state of the normal channel (now modified by BTX), since some analyses suggest that transitions from open to inactivated states are themselves voltage independent (Armstrong and Bezanilla, 1977; Aldrich et al., 1983).

TTX Binding to Single Channels

When TTX is added to the *cis* side of a bilayer containing a single BTX-activated Na^+ channel, distinct "blocking events" are observed (Fig. 7). These transient, zero-conductance states are much longer in duration than the closing events described above. We interpret each of these blocking events induced by TTX as being due to the binding of a single TTX molecule to the Na^+ channel, as previously suggested for saxitoxin (STX) (Krueger et al., 1983). The validity of this interpretation will be tested below.

In Fig. 8, dwell-time histograms for TTX-blocked and unblocked states are presented, after exclusion of rapid gating events, as described in Materials and Methods. As expected for a simple binding reaction, both blocked and unblocked

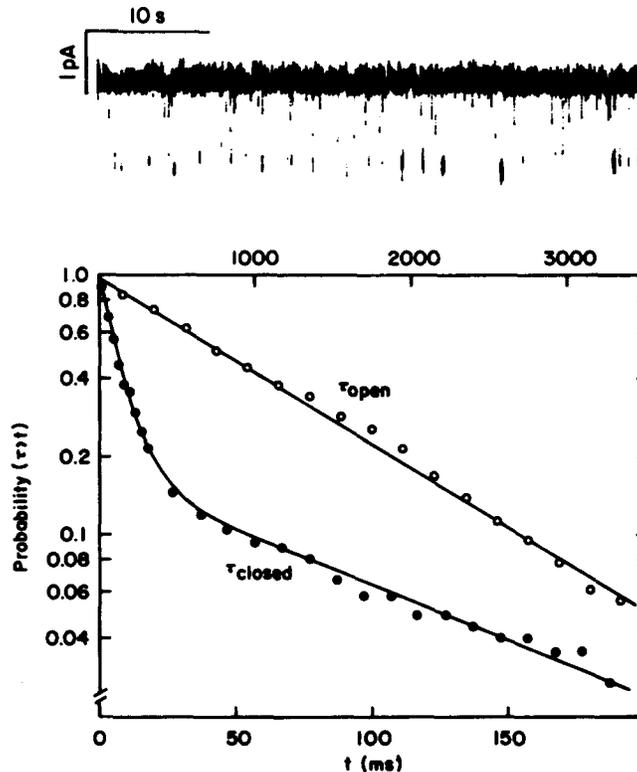


FIGURE 6. Dwell-time distributions at depolarized potentials. The upper trace shows a segment of the record (70 mV, 100-Hz filter) that was analyzed. Cumulative dwell-time histograms (lower plot) were constructed by computer after digitization of 306 s of analog input at 500 points/s. In all, 230 events were analyzed. The lower and upper time scales correspond to the closed and open state distributions, respectively. The time constant for the open state histogram was 1,170 ms, while the closed state distribution was fit by two exponentials with time constants 7.8 and 102 ms and amplitudes of 0.83 and 0.17, respectively.

states exhibit single-exponential probability distributions. We may therefore approximate the toxin binding reaction by the scheme:



According to this scheme, we group the open and closed states in a single state which we call the “unblocked” state. In the following analysis, we investigate toxin binding in the voltage range -60 to 60 mV, a range where the channel itself is nearly always open and shows no inherent voltage dependence.

The probability of TTX block is patently voltage dependent, as was originally shown for STX by Krueger et al. (1983). In Figs. 7 and 8, we see that at increasingly depolarized potentials, both blocking and unblocking rates change: the mean blocked time shortens, while the mean unblocked time lengthens.

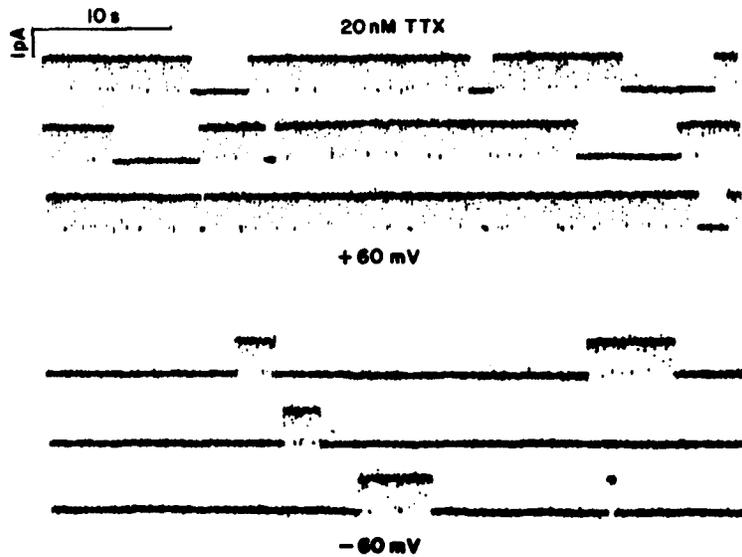


FIGURE 7. Discrete TTX block of a single Na^+ channel. The bilayer bath contained 20 nM TTX and 200 mM NaCl buffer. Each group of three traces represents one continuous record from the same bilayer.

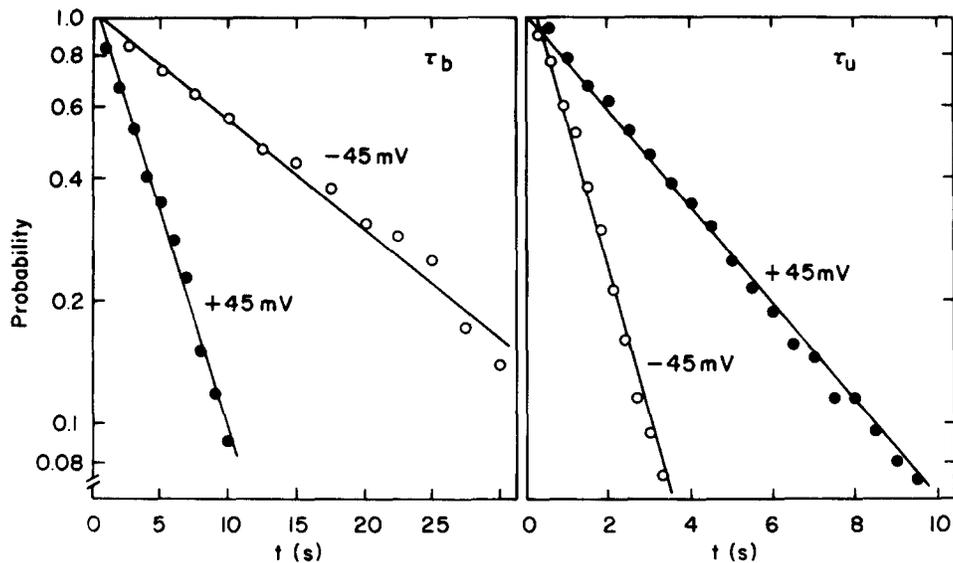


FIGURE 8. Dwell-time distributions in the presence of TTX. Histograms were tabulated from blocked (τ_b) or unblocked (τ_u) dwell times. The bilayer bath contained 100 nM TTX and 200 mM NaCl. The total number of events was 262 (45 mV) and 95 (-45 mV). Time constants for the fits are given in the text.

Table II further demonstrates this voltage dependence, and shows that the mean dwell times in the blocked and unblocked states are quantitatively reproducible from membrane to membrane. The table also confirms the equality of the mean and standard deviation of each sample, as expected for an exponential distribution. We also find that values derived from different experiments on different days and with different vesicle preparations agree well, and may be averaged as a single population. Table II also indicates that the mean unblocked time depends on TTX concentration, while the blocked time does not.

This last point is illustrated in more detail in Fig. 9. Here, we present the mean blocked times as a function of TTX concentration. The reciprocal unblocked time is directly proportional to TTX concentration, while the blocked time is independent of TTX. This is precisely the predicted behavior of Scheme I, and allows us to define a second-order rate constant of blocking, k_{ON} , and a first-order rate constant of unblocking, k_{OFF} :

$$k_{\text{ON}} = \{\tau_{\text{u}}[\text{TTX}]\}^{-1}; \quad (3a)$$

$$k_{\text{OFF}} = \tau_{\text{b}}^{-1}. \quad (3b)$$

Voltage Dependence of TTX Binding, and Competition by Na⁺

Fig. 10 shows the TTX binding rate constants measured over a range of voltages at two different Na⁺ concentrations. Both rate constants vary exponentially with voltage, with k_{OFF} increasing e-fold per 77 mV, and k_{ON} decreasing e-fold per 100 mV. We also see that raising the Na⁺ concentration decreases k_{ON} but leaves k_{OFF} unaffected. Moreover, the voltage dependence of these rate constants is not affected by Na⁺ concentration. These data are well described by:

$$k_{\text{ON}}(V) = k_{\text{ON}}(0) \exp(A_1 V); \quad (4a)$$

$$k_{\text{OFF}}(V) = k_{\text{OFF}}(0) \exp(A_{-1} V). \quad (4b)$$

Zero-voltage rate constants are plotted as a function of Na⁺ concentration in Fig. 11, and slope parameters A_1 and A_{-1} are given in Table III. One simple way

TABLE II
TTX Binding Statistics for Various Single Channel Bilayers

Bilayer	V	[TTX]	$\bar{\tau}_{\text{b}} \pm \text{SD} (n)$	$\bar{\tau}_{\text{u}} \pm \text{SD} (n)$
	mV	nM	s	s
1	+45	100	4.8±4.8 (49)	3.2±2.9 (52)
2	+45	100	5.1±5.4 (49)	3.9±3.2 (50)
3	+45	100	4.1±3.7 (51)	4.1±3.7 (55)
4	+45	100	4.4±3.8 (105)	3.6±3.6 (105)
1	-45	100	15±16 (55)	1.6±1.4 (61)
4	-45	100	18±14 (29)	1.3±1.0 (30)
5	-45	20	16±13 (32)	5.4±6.4 (32)
6	-45	20	15±20 (44)	7.6±7.2 (43)
7	-45	20	17±20 (27)	6.7±6.4 (28)

Single channel records were taken from different bilayers at the indicated voltage and TTX concentration. Individual blocked (τ_{b}) and unblocked (τ_{u}) events were measured and a mean ($\bar{\tau}$) and standard deviation (SD) were calculated from samples of n events.

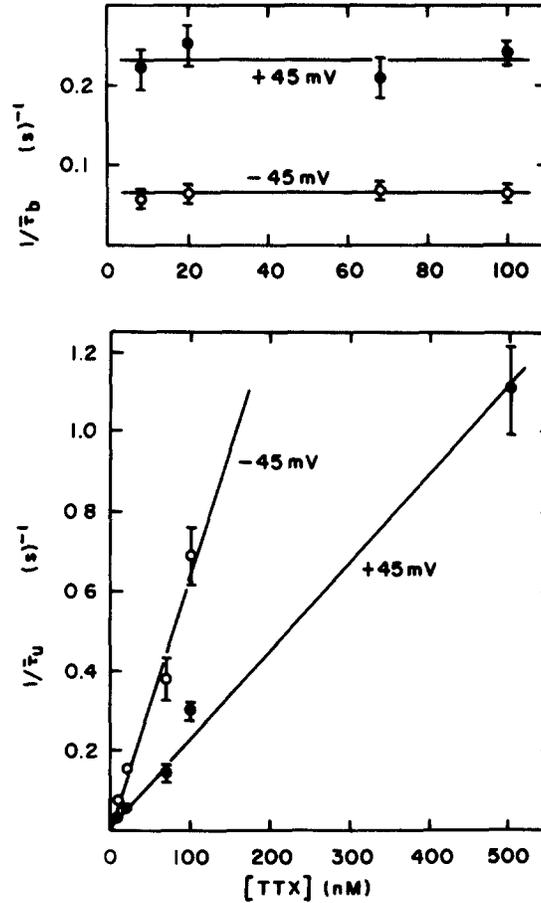


FIGURE 9. TTX dependence of reciprocal mean blocked and unblocked times. The mean and standard error of blocked or unblocked times were measured from single channel data at the indicated TTX concentration and voltage.

of explaining the Na^+ dependence of the rate constants is to assume that the binding of TTX to the channel is competitive with Na^+ ion:



where U and $\text{U}:\text{Na}^+$ represent unblocked states that are either free of or occupied by Na^+ at some crucial site. If Na^+ occupies this site, then TTX cannot bind to and block the channel. Scheme II thus predicts that the measured OFF rate of TTX will be independent of Na^+ , while the measured on rate will vary according to:

$$k_{\text{ON}}(\text{Na}^+) = k_{\text{ON}}^* (1 + [\text{Na}^+]/K_{\text{Na}})^{-1}, \quad (5)$$

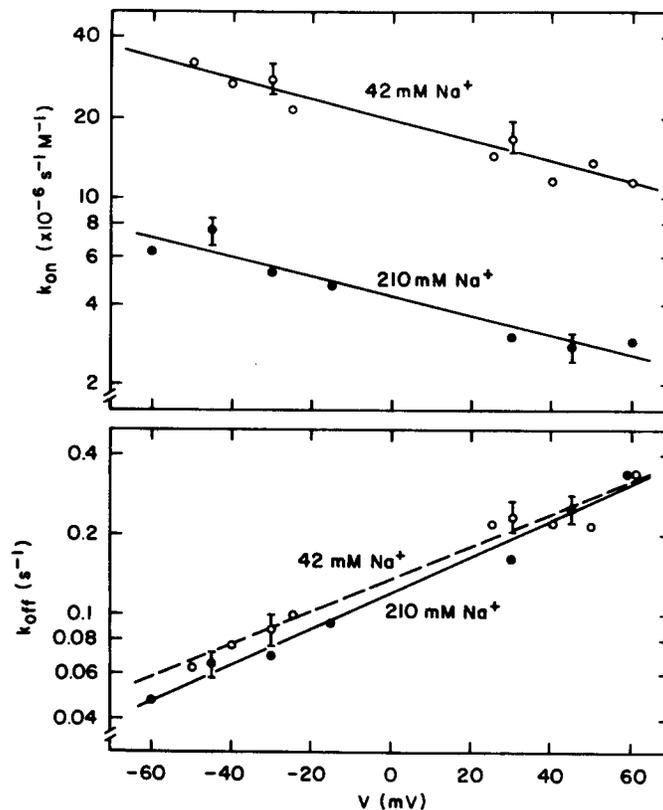


FIGURE 10. Voltage and Na⁺ dependence of TTX binding rate constants. Corrected rate constants at the indicated voltage and symmetrical Na⁺ concentration were measured from single channel data as described in the text. Solid lines are semilogarithmic least-squares fits. Zero-voltage rate constants from these fits are given in Fig. 9 and slope values are listed in Table III.

where k_{ON}^* is the ON rate constant for TTX in the limit of zero Na⁺, and K_{Na} is the equilibrium dissociation constant of Na⁺ binding to its site.

These predictions are in complete harmony with the data of Fig. 11. We find that k_{OFF} is constant at $0.12 \pm 0.02 \text{ s}^{-1}$, from 40 to 600 mM Na⁺. In addition, the reciprocal of k_{ON} varies linearly with [Na⁺] and yields a dissociation constant for Na⁺ of 32 mM and a TTX association rate constant of $3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ at zero Na⁺.

Having established competition between Na⁺ and TTX, we decided to investigate the accessibility of Na⁺ to its competitive binding site from either side of the bilayer. Fig. 12 shows the association rate constants of binding of TTX under conditions of asymmetrical Na⁺. The clear result emerges that TTX binding is competitively inhibited only by Na⁺ added to the *cis* side of the bilayer, i.e., on the same side as TTX itself. Na⁺ on the *trans* side has no effect on the rate constant of TTX binding. This conclusion holds regardless of the direction in which the Na⁺ current through the channel is actually flowing. As in Fig. 10,

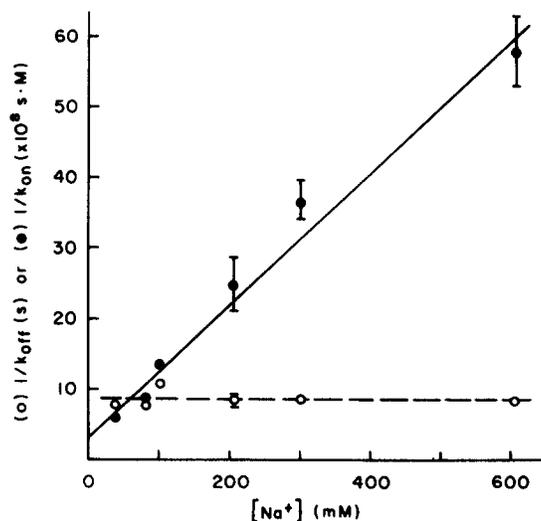


FIGURE 11. Na^+ dependence of zero-voltage rate constants for TTX binding. Rate constants at zero voltage were obtained from best fits of experimental data as shown in Fig. 10. The data are fit to the Na^+ competition model of Scheme II and Eq. 5.

only k_{ON} is affected by *cis* Na^+ ; k_{OFF} is independent of Na^+ under all conditions (not shown).

Na^+ Conductance as a Function of Na^+ Concentration

If conduction through the Na^+ channel involves at least one binding site, we would expect to observe saturation of the single channel conductance as Na^+ concentration is increased (Läuger, 1973; Hille, 1975*a*). The experiment of Fig. 13 shows that this is the case and that the conductance vs. concentration data are well described by a simple rectangular hyperbola over the range 3–600 mM Na^+ . A linear Scatchard plot of the data gives a $K_{1/2}$ for Na^+ of 8 mM, substantial-

TABLE III
Voltage Dependence of TTX Binding Rate Constants at Various Na^+ Concentrations

[Na^+]	Dissociation	Association
	A_{-1} mV^{-1}	A_1 mV^{-1}
42	0.014	-0.0091
80	0.013	-0.012
100	0.010	-0.0088
206	0.015	-0.0086
606	0.013	-0.012

TTX rate constants were measured as described in Figs. 10 and 11. A_{-1} and A_1 are slope parameters obtained from semilogarithmic least-square fits of the voltage dependence according to Eq. 3. The standard deviations of the A values were less than $\pm 0.002 \text{ mV}^{-1}$.

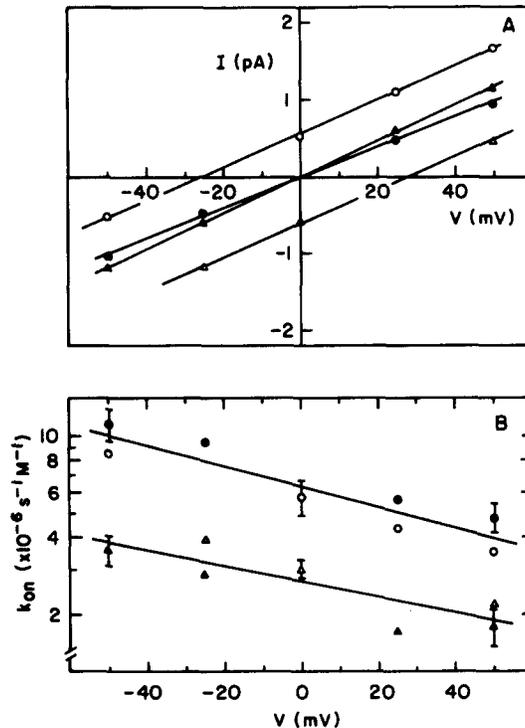


FIGURE 12. Effect of *cis* or *trans* Na⁺ on TTX association rate constants. Na⁺ channels were incorporated in the presence of 40 nM TTX and either symmetrical NaCl or a NaCl concentration gradient, where *cis* refers to the TTX-sensitive side: symmetrical 100 mM NaCl (●), symmetrical 300 mM NaCl (▲), 100 mM *cis*/300 mM *trans* NaCl (○), 300 mM *cis*/100 mM *trans* NaCl (△). (A) Measured single channel current as a function of applied voltage. The positive direction of Na⁺ current is defined as moving from the TTX-sensitive side to the TTX-insensitive side at positive voltages and symmetrical NaCl. (B) Measured TTX association rate constants at the indicated voltage. Solid lines correspond to least-squares fits of (●, ○) and (▲, △).

ly less than the apparent K_D of 32 mM for Na⁺ competition with TTX block (Fig. 11).

DISCUSSION

Behavior of Muscle Na⁺ Channels in Planar Bilayers

The basic properties of BTX-activated Na⁺ channels, as observed in planar bilayers, are similar to their characteristics in the native membrane. The voltage-dependent "activation" curve measured here (Fig. 2) is nearly identical to that measured for BTX-treated neuroblastoma (Huang et al., 1982) and frog node of Ranvier (Dubois et al., 1983), and the single channel gating kinetics in neuroblastoma (Huang et al., 1984) operate on the same time scale as seen here.

The conduction characteristics of rat muscle Na⁺ channels in planar bilayers

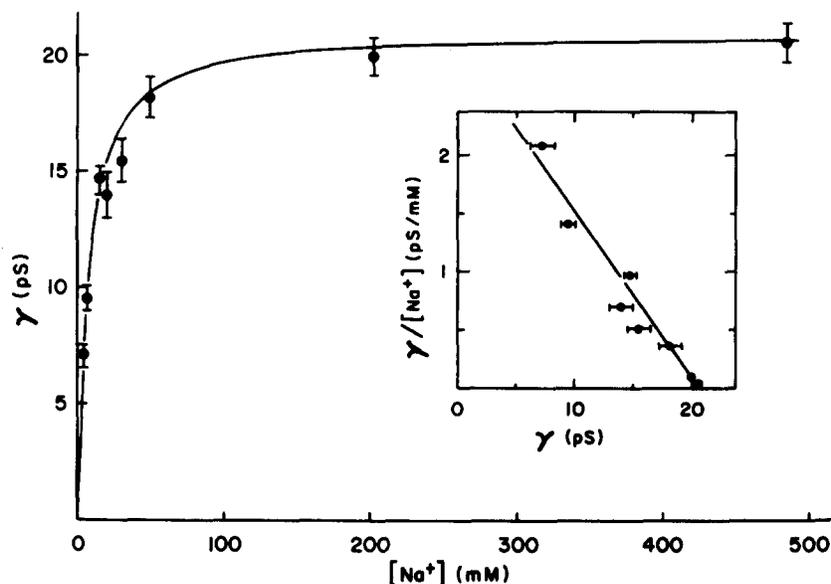


FIGURE 13. Single channel conductance as a function of Na^+ concentration. Unit conductance (γ) was measured as the zero-voltage slope of single channel current-voltage data at the indicated symmetrical Na^+ concentration. A Langmuir isotherm is drawn through the data as obtained from the linear best fit of a Scatchard plot (inset). For this fit, $\gamma_{\text{max}} = 21$ pS, and $K_{1/2} = 8$ mM.

are also similar to those of Na^+ channels observed under physiological conditions. It is difficult to make a detailed comparison, since our ionic conditions are simpler than those required in physiological experiments. However, our value of single channel conductance of 20 pS at 200 mM Na^+ is the same as that of the normal Na^+ channel in rat myotubes (Patlak and Horn, 1982), after correction for temperature differences (Horn et al., 1984; Yamamoto et al., 1984). The single channel conductance measured here with rat muscle channels is smaller than the value of 30 pS at 500 mM NaCl reported for BTX-activated Na^+ channels from rat brain in planar bilayers (French et al., 1984). This discrepancy appears to be due to a specific difference between the two tissues; under identical conditions of 200 mM NaCl, we have found (data not shown) that the unit conductance is significantly higher for the rat brain channel (24 pS) than for the rat muscle channel (20 pS).

The ionic selectivity found here is also consistent with the known properties of Na^+ channels. As is the case in frog node of Ranvier (Hille, 1971; Chandler and Meves, 1965), Li^+ displays a nearly identical biionic permeability as Na^+ but a distinctly lower conductance. The ionic selectivity sequence we observe (Table I) is the same as that of normal and BTX-modified Na^+ channels (Hille, 1971; Huang et al., 1979). Our measured permeability ratio of 0.07 for $P_{\text{K}}/P_{\text{Na}}$ is lower than that found in BTX-treated neuroblastoma (Huang et al., 1979), but this ratio may depend on ionic conditions.

While we expected to observe saturation of single channel conductance with

Na⁺ concentration (Hille, 1975*b*), we were surprised by the high apparent affinity of the conduction pathway for Na⁺ ($K_{1/2} = 8$ mM). It is unlikely that this high affinity is due to local surface charges near the channel mouth, since we are working with neutral phospholipid membranes, and since an electrostatic effect of this kind would be expected to cause a large deviation from linearity of the Scatchard plot of the data (Bell and Miller, 1984).

Under slightly different ionic conditions, Hansen-Bay and Strichartz (1980) measured a K_D of 28 nM (corrected to 24°C) for TTX binding to whole rat muscle, in agreement with our zero-voltage value of 27 nM at 200 mM NaCl. Therefore, by the criteria of gating, conductance, selectivity, and pharmacology, we conclude that the channel we observe in planar bilayers is the BTX-activated Na⁺ channel in a state not discernibly different from that in its native membrane.

Is TTX Binding Voltage Dependent in Cells?

Despite the similarities noted above, the following question remains open: why has the voltage dependence of TTX blocking not been observed in studies on toxin binding to cell membranes? The evidence relating to this question has been reviewed by French et al. (1984), who proposed that the voltage dependence of toxin block may arise from voltage-dependent binding of ions such as Ca²⁺ or Na⁺. Our results eliminate a requirement for Ca²⁺, since the same voltage dependence was observed here in the presence of EDTA. A variation in the Na⁺ concentration from 40 to 600 mM also does not significantly affect the voltage dependence (Table III).

We are not convinced that a discrepancy on this point actually exists. The lack of voltage-dependent binding in cell membrane systems may be due to other factors, such as a failure to attain binding equilibrium in voltage pulse studies, or the possibility that binding of toxins to nonconducting states of the channel (the resting and inactivated states) may be independent of voltage.

Other Possible Interpretations of the Na⁺ Effect

We have chosen to explain the relief of TTX block by Na⁺ by the simple competitive model of Scheme II. This interpretation is not unique, but it requires the fewest assumptions. For instance, we could assume that TTX and Na⁺ bind to different sites simultaneously, with TTX binding to the Na⁺ form having lower affinity. This noncompetitive model requires the mean unblocked time to saturate with increasing Na⁺, as the equilibrium is shifted completely to the Na⁺-occupied states. However, no such saturation is observed up to 600 mM Na⁺ (Fig. 11). Furthermore, such a model would fortuitously require that TTX dissociation rates be the same for Na⁺-free and Na⁺-bound states, in order for k_{OFF} to be independent of Na⁺ (Fig. 11).

Because Na⁺ clearly competes with TTX for binding, we wished to test the possibility that the voltage dependence of TTX binding is merely a consequence of a voltage-dependent Na⁺ binding, i.e., that the TTX binding reaction is not intrinsically voltage dependent. Unfortunately, the scatter of our data does not allow the direct determination of the voltage dependence of the Na⁺ binding constant. However, two observations argue strongly that the observed TTX

voltage dependence does not arise from Na^+ binding. The first is that the voltage dependence of TTX binding does not vary with Na^+ concentration (Fig. 10). The second is that both dissociation and association rate constants of TTX are voltage dependent, while only the association rate constant is Na^+ dependent. We therefore conclude that voltage dependence is an inherent property of the TTX binding reaction, and not merely a result of coupling to voltage-dependent Na^+ binding.

Since we have varied ionic strength as well as Na^+ concentration, one might argue that the enhanced k_{ON} rate of the TTX cation at low NaCl is due to the electrostatic effect of a negatively charged protein, i.e., that the local negative surface potential at the binding site increases as the ionic strength is lowered. But if the Na^+ effect were purely electrostatic, again one would expect k_{ON} to saturate at high salt, where charges are well screened, in contradiction to the results of Fig. 11.

While the expectations of these alternative interpretations are incompatible with available data, the simple occupancy effect due to pure competition receives additional support from TTX and STX binding data. In studies using radioactive toxins, it has been shown for three different tissues that the apparent K_{D} for toxin binding is a linear function of the concentration of monovalent alkali cations (Reed and Raftery, 1976; Weigele and Barchi, 1978; Barchi and Weigele, 1979). Moreover, the measured K_{D} of Na^+ competition for STX binding in rat muscle is 37 mM in experiments done at constant ionic strength (Barchi and Weigele, 1979), a value similar to ours obtained by varying ionic strength.

If single site competition is the actual mechanism for the Na^+ effect, we might ask how a single Na ion could prevent the binding of much larger toxins capable of many different types of chemical interactions in the binding site. The simplest mechanism would be the prevention by Na^+ of a single, but absolutely required, ionic interaction with the toxin guanidinium. This idea is consistent with the effect of pH on toxin binding. Protons also appear to inhibit toxin binding competitively with a pK_{a} of 5.3 (Henderson et al., 1974; Reed and Raftery, 1976).

Does TTX Plug the Pore?

While physical occlusion of the channel lumen by the toxin is a simple and easily visualized picture of the blocking interaction, an alternative allosteric model can be considered, in which toxin binds far away from the pore and indirectly leads to channel closing. Presently, no compelling evidence exists to rule out either mechanism. Because of the economy of the occlusion mechanism, however, we prefer to retain this model as a basis of discussion and focus on the implications of our results within the occlusion paradigm. The question of TTX block then becomes one of degree: how deeply does TTX enter the pore?

In an extensive survey of blockade of K^+ channels by cations, French and Shoukimas (1985) distinguished "deep" and "shallow" blockers by several criteria. Deep blockers exhibit a substantial voltage dependence of binding. They also display phenomena suggestive of binding within the ion conduction pathway, such as permeation at high voltage or displacement by permeant ions added to

the opposite side of the membrane. Shallow blockers, on the other hand, exhibit only slight voltage dependence of binding and no interaction with conducting ions added *trans* to the blocking side. Since guanidinium ions permeate the Na⁺ channel (Hille, 1971), it is pertinent to ask whether guanidinium group toxins like TTX exhibit deep or shallow blocking behavior.

Results of the past several years have cast doubt on the original picture of deep block by TTX at the channel's selectivity filter. One such indication is that trimethyloxonium (TMO), a carboxyl methylating reagent, renders the Na⁺ channel insensitive to guanidinium toxins but leaves its ionic selectivity and block by protons largely unaffected (Spalding, 1980). Thus, the TMO-sensitive carboxyl, which presumably pairs with the toxin guanidinium group, cannot be part of the intimate selectivity region of the channel. It has also been shown (Huang et al., 1979) that two different neuroblastoma cell lines have very different TTX dissociation constants (18 and 1,500 nM), but exhibit nearly identical ion selectivity properties.

The gross characteristics of toxin blockade documented here, a voltage-dependent binding reaction in which the association rate constant is competitively inhibited by Na⁺, would seem to favor a model in which the toxin enters the pore deeply. Indeed, such evidence is normally considered sufficient for a plugging mechanism (Miller, 1982). However, the detailed aspects of both Na⁺ competition and of TTX voltage dependence argue against deep penetration of the toxin into the channel. Our results, like those of other groups (Reed and Raftery, 1976; Barchi and Weigele, 1979), indicate that TTX and Na⁺ bind competitively to the channel protein. The new result here is that Na⁺ is an effective competitor only when added to the same side of the channel as TTX. If Na⁺ were competing for occupancy of a site deep within the conduction pathway, we would expect it to displace TTX from either side of the channel. This is clearly not the case (Fig. 12), regardless of the direction of Na⁺ current flowing through the channel. In addition, the observed difference between the $K_{1/2}$ for Na⁺ saturation of channel conductance (8 mM) and the K_D for Na⁺ competition (32 mM) suggests that these represent different sites.

In summary, the results presented here on Na⁺ competition do not provide the least encouragement for the notion that the TTX molecule plugs the pore by entering it deeply. In the following paper (Moczydlowski et al., 1984), we show that the voltage dependence of the toxin binding reaction also fails to support this notion. Under the assumption that the toxins inhibit by physically occluding the pore, we favor the idea that this occlusion occurs at a superficial region near the channel's entryway. At such an extreme location, the toxin binding site would neither sense the occupancy of the conduction pathway by Na⁺ nor reside within the applied electric field.

What, then, is the significance of Na⁺ competition for TTX binding? One possibility is that there is no significance at all, that Na⁺ happens to bind to a group required for stabilization of the toxin on the channel protein. A more interesting possibility is that this Na⁺ site, at the outer regions of the pore mouth, may function as a "pre-filter" in conduction, i.e., that it might actually be involved in Na⁺ conduction, though not deeply within the channel. Such a pre-filter might

act to enhance the rates of cation entry into deeper regions of the pore. In fact, direct binding studies of guanidinium toxins have shown that the alkali metal cations compete with toxin binding, with a "conduction-like" selectivity sequence: $\text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+$ (Reed and Raftery, 1976; Barchi and Weigele, 1979). However, the selectivity of competition is much weaker than the selectivity of conduction, with Na^+ being only twice as effective a competitor than K^+ and only fourfold more effective than Rb^+ and Cs^+ . Such a weakly selective Na^+ site would not ultimately determine the selectivity of ionic current, but may contribute to flux rates and ionic selectivity. We picture this site in rapid equilibrium with the solution it faces, so that Na ions flowing from the *trans* side of the channel would not compete with TTX binding from the *cis* side. This proposal would also explain why TMO modification of this carboxyl reduces Na^+ conductance (Sigworth and Spalding, 1980). In conclusion, our current working hypothesis for TTX block is similar to that proposed by Kao and Walker (1982), where the toxin molecule bears more resemblance in its blocking action to a lid on a funnel than to a cork in a bottle.

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