Single Na⁺ Channels Activated by Veratridine and Batrachotoxin

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ABSTRACT Voltage-sensitive Na⁺ channels from rat skeletal muscle plasma membrane vesicles were inserted into planar lipid bilayers in the presence of either of the alkaloid toxins veratridine (VT) or batrachotoxin (BTX). Both of these toxins are known to cause persistent activation of Na⁺ channels. With BTX as the channel activator, single channels remain open nearly all the time. Channels activated with VT open and close on a time scale of 1–10 s. Increasing the VT concentration enhances the probability of channel opening, primarily by increasing the rate constant of opening. The kinetics and voltage dependence of channel block by 21-sulfo-11-α-hydroxysaxitoxin are identical for VT and BTX, as is the ionic selectivity sequence determined by bi-ionic reversal potential ($Na^+ \sim Li^+ > K^+ > Rb^+ > Cs^+$). However, there are striking quantitative differences in open channel conduction for channels in the presence of the two activators. Under symmetrical solution conditions, the single channel conductance for Na⁺ is about twice as high with BTX as with VT. Furthermore, the symmetrical solution single channel conductances show a different selectivity for BTX ($Na^+ > Li^+ > K^+$) than for VT ($Na^+ > K^+ > Li^+$). Open channel current-voltage curves in symmetrical Na⁺ and Li⁺ are roughly linear, while those in symmetrical K⁺ are inwardly rectifying. Na⁺ currents are blocked asymmetrically by K⁺ with both BTX and VT, but the voltage dependence of K⁺ block is stronger with BTX than with VT. The results show that the alkaloid neurotoxins not only alter the gating process of the Na⁺ channel, but also affect the structure of the open channel. We further conclude that the rate-determining step for conduction by Na⁺ does not occur at the channel's "selectivity filter," where poorly permeating ions like K⁺ are excluded.

INTRODUCTION

The action potential in many excitable cells is propagated by the opening and spontaneous inactivation of the voltage-sensitive Na⁺ channel. Because this channel is essential to electrical conduction in nerve and muscle, it is a favorite target for natural toxins that have evolved to destroy its normal function and thus prevent integrated neuronal activity. One class of such toxins, the alkaloid

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neurotoxins, includes veratridine (VT) and batrachotoxin (BTX). These toxins act by binding to the Na^+ channel and preventing, or vastly slowing, its normal inactivation, an action that leads to hyperexcitable axons.

The mode of action of BTX is now quite well understood. Recent electrophysiological and single channel studies have shown that this toxin shifts the Na^+ channel's normal voltage-dependent activation curve toward more hyperpolarized potentials, and totally prevents inactivation from occurring on a time scale as long as hours (Huang et al., 1982; Quandt and Narahashi, 1982; Dubois et al., 1983; Krueger et al., 1983; French et al., 1984; Moczydlowski et al., 1984a). The action of VT is not as well documented. It is known that VT is less potent than BTX and that its full effect is much weaker than that of BTX (Catterall, 1975, 1977; Tamkun and Catterall, 1981; Weigele and Barchi, 1982). However, the effect of VT on Na^+ channels has not previously been described at the single channel level.

In this report, we study single Na^+ channels activated by VT and compare them with channels activated by BTX. The effects of the activators on single Na^+ channels are remarkably different. Not only do the toxins activate Na^+ channels differently, as one might expect from their known cellular effects, but the ion conduction behavior of the open channel is dependent on the activator. We conclude that the alkaloid neurotoxins not only act on the activation and inactivation processes of the Na^+ channel, but also profoundly modify the structure of the open pore. A preliminary report of this work has been presented (Garber and Miller, 1985).

MATERIALS AND METHODS

Chemicals and Toxins

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from egg yolk or bovine brain were purchased from Avanti Polar Lipids (Birmingham, AL) and stored in chloroform under N_2 at $-70°C$. The acetate salts of Li^+, Rb^+, and Cs^+ were obtained from Alpha Inorganics (Danvers, MA). VT was supplied by Dr. Joshua Zimmerberg or was purchased from Sigma Chemical Co. (St. Louis, MO). The purity of VT samples was confirmed by reverse-phase high-pressure liquid chromatography (Holan et al., 1984). BTX was a gift of Dr. John Daly. Tetrodotoxin (TTX) was obtained from Calbiochem-Behring (San Diego, CA), and 21-sulfo-11-α-hydroxysaxitoxin (C1) was supplied by Dr. Sherwood Hall. Other chemicals were reagent-grade samples obtained from Fisher Chemical Co. (Fair Lawn, NJ).

Plasma membrane vesicles were prepared from rat skeletal muscle as described (Moczydlowski and Latorre, 1983), except that the Ca^{++}-loading step was omitted and the vesicles were collected on a cushion of 30% (wt/vol) sucrose. Vesicles were stored in 300 mM sucrose at $-70°C$.

Planar Bilayers and Insertion of Na^+ Channels

The planar bilayer setup and its associated electronics have been described (Hanke and Miller, 1983). A capacitance-compensation circuit (Sigworth, 1983) was added to cancel the capacitive current generated during voltage ramps. All experiments were carried out by reconstituting vesicles containing Na^+ channels from rat muscle into essentially solvent-free phospholipid bilayers formed by folding air-water interface monolayers over a hole

(100–150 μm diam) in a Teflon partition (Montal and Mueller, 1972). Lipid monolayers were formed by adding 5 μl of a solution of 4 mM PE plus 1 mM PC in *n*-pentane to the surface of the aqueous solution on each side of the partition. The partition was first primed with a solution of 4% hexadecane in pentane. The pentane was allowed to evaporate for several minutes before the bilayer was formed by raising the level of the solutions over the hole.

The standard aqueous solution used was 200 mM Na-acetate, 10 mM MOPS, and 0.1 mM EDTA, adjusted to pH 7.4 with NaOH. In some experiments, the concentrations of Na-acetate were varied as indicated; in experiments using other cations to replace Na^+, the acetate salts were always used and the pH was adjusted with the hydroxide of the appropriate cation. For experiments at low ionic strength, only 5 mM MOPS was used and the buffers contained 0.5 mM EDTA in addition to 0.1 mM EGTA. In all experiments, the total concentration of Na^+ is reported, including Na^+ from the Na-acetate, as well as from the NaOH used to neutralize the acid forms of the MOPS, EDTA, and EGTA. Acetate-containing solutions were used in these experiments because of a much higher success rate of obtaining clean single channel insertion events than with Cl^--containing solutions. Pilot experiments show that the behavior to be described, however, is identical to that in Cl^- media.

The incorporation of single channels into the bilayer was induced by adding plasma membrane vesicles (~5 μg/ml) to one side of the bilayer (defined as the *cis* side) while maintaining a holding voltage of 50 mV, or by applying a voltage ramp from −100 to +100 mV, with either VT (50 μM) or BTX (0.2 μM) present in the aqueous solution. In most experiments, channels were inserted under symmetrical solution conditions. For experiments at ionic strengths lower than 50 mM, channels were first inserted at 200 mM salt, and then the appropriate experimental solutions were introduced by perfusion of the aqueous chambers. Channel incorporation usually occurred within 30 min and was recognized by the appearance of unitary conductance steps of ~10 pS (VT present) or 20 pS (BTX present). Channels were usually inserted with the TTX-sensitive side presented to the *cis* side of the bilayer, but in each case the channel's orientation was determined by the voltage dependence of its gating or by its sensitivity to added TTX. In some experiments, channels with the incorrect orientation were eliminated by including a high concentration (1 μM) of TTX on one side of the bilayer. All voltages are reported according to the electrophysiological convention, with the TTX-sensitive side of the channel defined as zero voltage.

Storage and Analysis of Single Channel Records

Records obtained from bilayers maintained at constant applied voltage were filtered at a cutoff frequency of 50–400 Hz and collected on FM tape for later analysis by hand. For determining open channel current-voltage (*I-V*) curves, repetitive voltage ramps were applied to the bilayer, using a laboratory computer system (Indec Systems, Sunnyvale, CA). Typically, voltage was linearly varied from −100 to +100 mV over 2.5 s, and current was sampled at 100 points/s and stored by the computer. For a given *I-V* curve, 64 such ramps were collected. Later, the records were reviewed, and open and closed intervals were averaged separately to obtain the open channel *I-V* curve. An example of this procedure is presented in Fig. 1 to demonstrate that the *I-V* relation can be reliably obtained.

Reversal potentials under bi-ionic conditions were always measured using the voltage-ramp method, which allows direct determination of the zero-current voltage within 6 mV, at worst. The recorded values of reversal potential were corrected for liquid junction potential asymmetries via the Henderson equation, and were in the range 3–6 mV. We

always checked, before and after each experiment, that asymmetry potentials (measured with no membrane across the septum) were <2 mV.

Analysis of C1 Blocking Kinetics

In one series of experiments, we determined the rates of channel blocking and unblocking of a saxitoxin (STX) derivative, C1, by analyzing the rapid block induced by this toxin, as has been done before for discrete blocking of single channels (Neher and Steinbach, 1978; Miller, 1982). In this case, there were two complications that needed to be corrected.
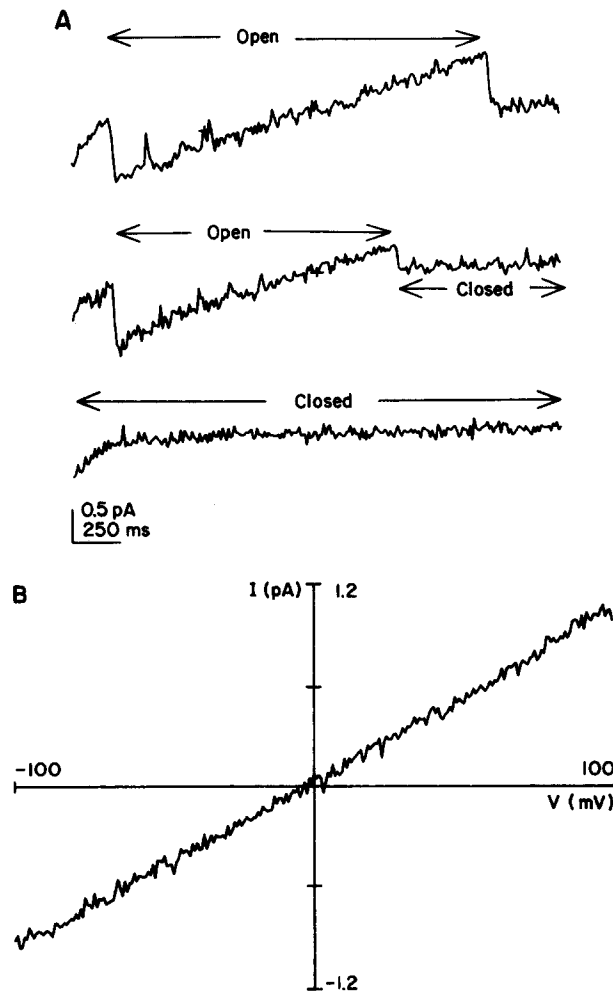
FIGURE 1.   Voltage-ramp method for I-V curve determination. Single Na^+ channels openings were observed in the presence of VT (35 μM) with a linear voltage ramp (-100 to 100 mV over 2.5 s) applied across the bilayer. In A, three successive traces are displayed, with the open and closed sections marked. The final I-V curve in B was produced from averaging open segments and subtracting averaged closed segments. I-V curves calculated in this way agreed to within 5% with currents measured at constant holding voltages.

First, signal-to-noise considerations forced us to filter the channel records at 50–100 Hz, so blocking events shorter than ~10 ms were not detected. Second, the spontaneous, long-lived closings of the VT-activated channel had to be excluded from the population of short-lived toxin-blocking events; thus, we considered any nonconducting event longer than 1 s a closing, and any shorter event was considered a blocking. Assuming an exponential distribution for the blocked times (as we have confirmed), then the true mean blocked time, τ_b, is given in terms of the observed mean blocked time, τ_{obs}, according to the approach of Neher and Steinbach (1978):

$$\tau_b = \tau_{obs} - \frac{L\exp(-L/\tau_b) - U\exp(-U/\tau_b)}{\exp(-L/\tau_b) - \exp(-U/\tau_b)},$$

where L and U are lower and upper cutoff times, respectively. This equation was solved iteratively, with $L = 0.013$ s and $U = 1$ s.

RESULTS

VT-activated Na⁺ Channels

The voltage-dependent Na^+ channel is normally activated in response to a depolarizing voltage pulse. Once activated, the channel spontaneously inactivates after ~1 ms, much too rapidly to be observed with the planar bilayer system used here. In the presence of VT, however, the addition of plasma membrane vesicles in Na^+-containing solutions consistently induced the appearance of the channels shown in Fig. 2. At 50 mV, the channel remained open for several seconds on average before closing. At −50 mV, the channel spent more of its time closed, and stayed for a shorter time, on average, in the open state.

The channels shown in Fig. 2 appear very different from single Na^+ channels activated by BTX, which have been extensively characterized by several laboratories (Krueger et al., 1983; French et al., 1984; Huang et al., 1984; Moczyd-lowski et al., 1984a, b). Whereas BTX-activated Na^+ channels remained open almost always at voltages positive to −60 mV, the channels we observed in the presence of VT opened only infrequently. Furthermore, the VT-induced channels displayed only about half of the open state conductance of BTX-activated Na^+ channels. The question therefore arises whether these are Na^+ channels at all. We will present two lines of evidence arguing that these are indeed voltage-dependent Na^+ channels: ionic selectivity, and inhibition by guanidinium toxins.

The VT-induced channels are selective for Na^+. Fig. 3 illustrates open channel *I-V* curves under bi-ionic conditions, with Na^+ on one side of the bilayer and either K^+ or Rb^+ on the other. The reversal potentials for all the group IA cations are summarized in Table I. The reversal potential for K^+ measured in VT-activated frog node and muscle agree well with what we report here (Naumov et al., 1979; McKinney, 1984). The permeability sequence derived from these data, $P_{Na} \sim P_{Li} > P_K > P_{Rb} > P_{Cs}$, is the same as that of the unmodified Na^+ channel (Hille, 1972).

The ability of externally applied guanidinium toxins, such as TTX and STX, to block Na^+ current is a classic diagnostic test for the voltage-sensitive Na^+ channel. The VT-induced Na^+-selective channel is sensitive to TTX from one side of the bilayer, as Fig. 4 shows. In this experiment, we studied a bilayer

containing at least four VT-induced channels. The addition of a high concentration of TTX to the *trans* side of the bilayer was without effect, but a similar addition on the *cis* side abolished the appearance of channels. The effect of TTX is readily reversed by perfusion of the aqueous solution (data not shown). It would be desirable to quantify this result by measuring the association and dissociation kinetics of guanidinium toxin with the channel, as others have done
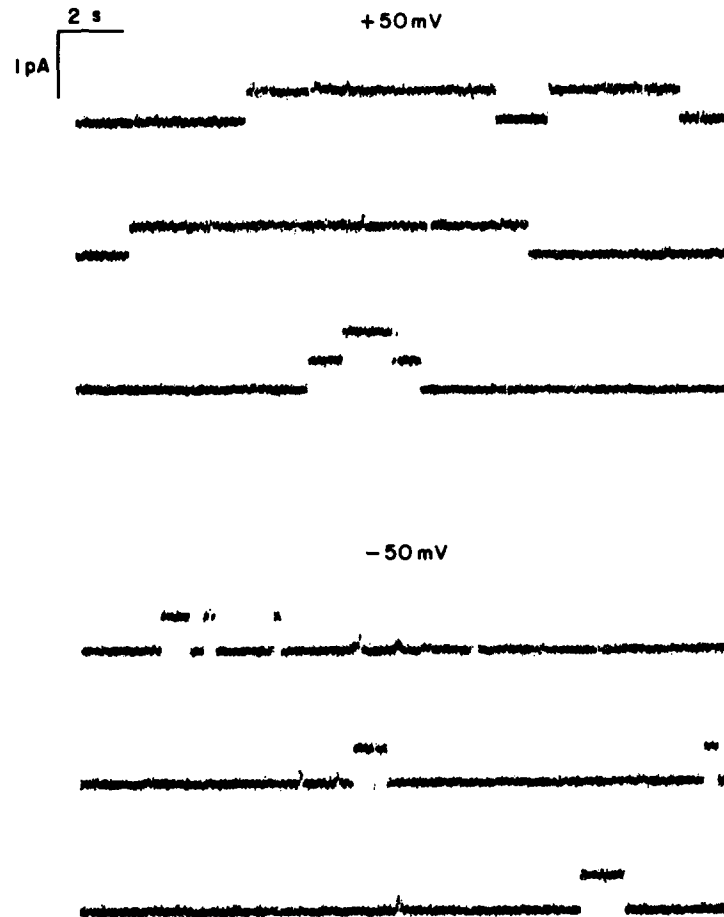

FIGURE 2.   Single VT-activated channels. A bilayer containing at least two VT-activated channels was observed at 50 or −50 mV in the presence of 35 μM VT, under symmetrical 200 mM Na$^+$ conditions, as described in Materials and Methods. In all traces, opening is upward.

with the BTX-activated channel (Krueger et al., 1983; French et al., 1984; Moczydlowski et al., 1984*a*, *b*). Unfortunately, it is impossible to measure directly the block by TTX or STX of the VT-induced channel, since the characteristic long closings of this channel are much longer than the toxin-blocking events.

We can, however, use C1 to circumvent this difficulty. This low-affinity STX derivative has an average block time that is much shorter (~100 ms) than the
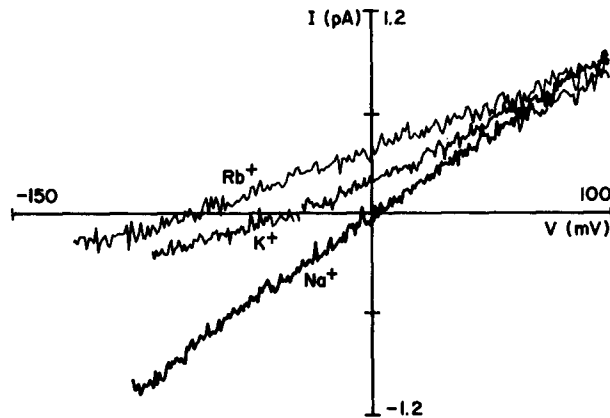
FIGURE 3.   VT-activated channels are Na^+ selective. Open channel *I-V* curves were measured by the voltage-ramp method under bi-ionic conditions, with 200 mM Na^+ solution on the internal side of the bilayer, and 200 mM Na^+, K^+, or Rb^+ solutions, as indicated, on the external side. The reversal potentials are reported in Table I.

average open time of the VT-induced channel (Moczydlowski et al., 1984*b*). Fig. 5 shows the rapid blocking by Cl of both VT- and BTX-activated Na^+ channels. This toxin induces brief nonconducting states in both of these channels. We know that in the BTX-activated channel, these states represent the binding of single molecules of Cl toxin to the channel (Moczydlowski et al., 1984*b*). Fig. 5 shows that a similar effect of Cl is seen with VT-activated Na^+ channels. The average blocked and unblocked times as a function of voltage for both BTX- and VT-induced channels are plotted in Fig. 6. The figure shows that the toxin-

TABLE I

Bi-ionic Reversal Potentials of VT- and BTX-modified Na^+ Channels

Toxin	Ion	57 mM	206 mM	504 mM
		mV	*mV*	*mV*
VT	Li	0±4	−5±4	−8±5
	K	−30±5	−29±4	−25±6
	Rb	ND*	−63±5	ND
	Cs	ND	< −75	ND
BTX	Li	−4±4	−3±2	−3±3
	K	−53±5	−57±4	−51±4
	Rb	ND	< −88	ND
	Cs	ND	< −95	ND

Single channel reversal potentials were determined under bi-ionic conditions at the indicated concentration of test cation. In all experiments, Na^+ was the reference cation and was at the same concentration as the test cation. All experiments were performed in the antiphysiological orientation, with Na^+ present internally and the test cation present externally. Values represent the means and standard errors of 4–10 separate determinations, in at least three different bilayers.
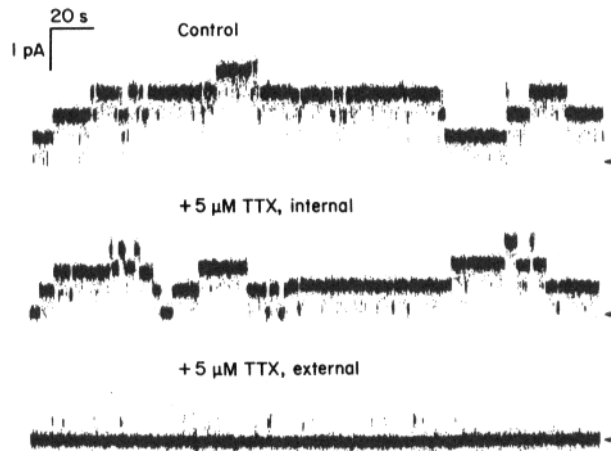
* Not determined.

FIGURE 4. TTX blocks VT-activated channels. A bilayer containing at least four VT-activated channels was observed at 50 mV, as in Fig. 2. After recording a control trace (upper trace), TTX (5 μM) was added to the internal side of the bilayer (center trace). The same amount of TTX was then added to the external side of the bilayer (lower trace). The arrows mark the level of zero current.
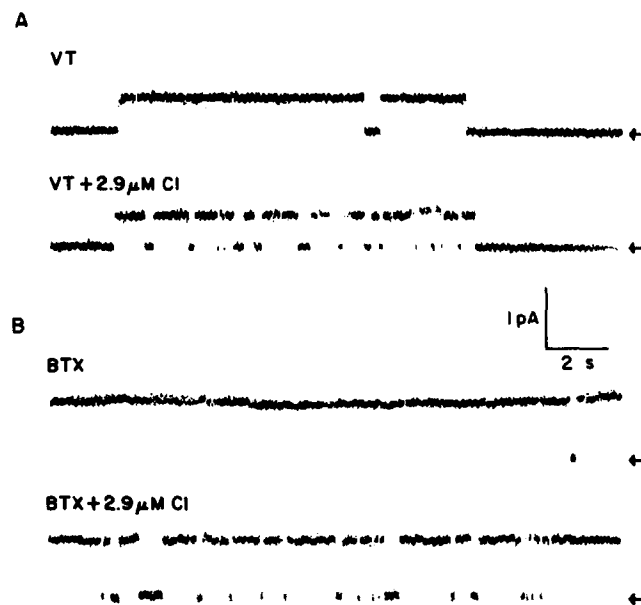

FIGURE 5. Block of alkaloid-modified Na^+ channels by the STX derivative C1. Single Na^+ channel openings were observed in the presence or absence of C1 added to the external solution to a final concentration of 2.9 μM. The arrows indicate the level of zero current. The holding voltage was 50 mV, and records were filtered at 100 Hz. The block by C1 is manifested by brief nonconducting events. Channels were observed in the presence of 35 μM VT (A) or 0.2 μM BTX (B).

blocking kinetics are quantitatively identical with VT or BTX as the channel activator, across the voltage range studied.

The Na^+ selectivity of the currents and the identical guanidinium toxin sensitivity of the channels together lead us to the conclusion that the channels observed in the presence of VT are voltage-gated Na^+ channels, and we will henceforth refer to them as such. The remainder of this study seeks to compare the properties of Na^+ channels in the presence of these two different activators.

Channel Activation

Records of VT- and BTX-activated Na^+ channels (Fig. 5) show the obvious qualitative difference between these two activators. BTX-modified channels
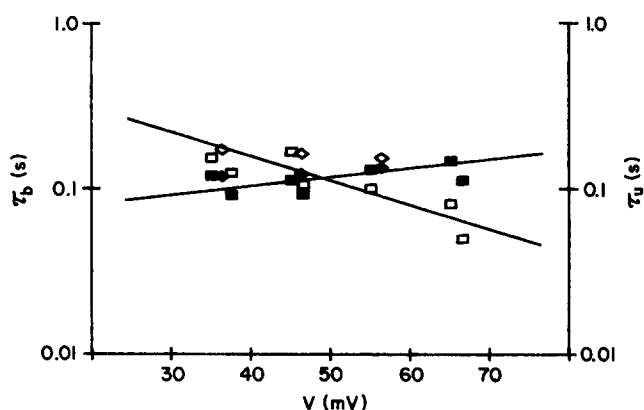

FIGURE 6. Cl blocking kinetics of Na^+ channels activated by VT or BTX. Single Na^+ channel openings were examined in the presence of 6 μM Cl, and the rapid blocking and unblocking kinetics induced by this STX analogue were analyzed as a function of holding voltage as described in Materials and Methods. Mean times in the blocked (τ_b, open symbols) and unblocked (τ_u, filled symbols) states are reported. Squares: VT used as the activator; diamonds: BTX used as the activator. Each point represents the average of 75–200 events. Standard errors did not exceed ±10%. Data were compiled from four bilayers. The solid lines are least-squares fits to the points. The corresponding association and dissociation rate constants are fitted by: $k_{on} = 2.5 \times 10^6 \exp(-0.012V)$ M^{-1} s^{-1} and $k_{off} = 3.4 \exp(0.015V)$ s^{-1}, where V is the applied voltage in millivolts.

almost never closed, remaining open >95% of the time; the closings that were observed were brief, on the order of a few milliseconds. In the presence of VT, in contrast, the channel was often closed and openings were brief, on the order of a few seconds. As the VT concentration was increased (Fig. 7A), we observed more frequent openings, and it became obvious that numerous channels were present in the bilayer. Even at high concentrations (>100 μM), where the VT effect is maximal, channels continued to open and close frequently. This observation is a direct confirmation at the single channel level of the previous suggestion that even at maximal concentrations of VT, the channel still spends

a substantial fraction of its time in a closed state (Catterall, 1975, 1977; Weigele and Barchi, 1982).

Experiments in which the VT concentration was varied as in Fig. 7A showed that the probability of observing the open state increased in the range of 5–200 μM VT, and that the major effect of the toxin was to reduce the average time spent in the nonconducting state; no discernible effect of VT concentration has been found on the average open time (data not shown). Unfortunately, we have
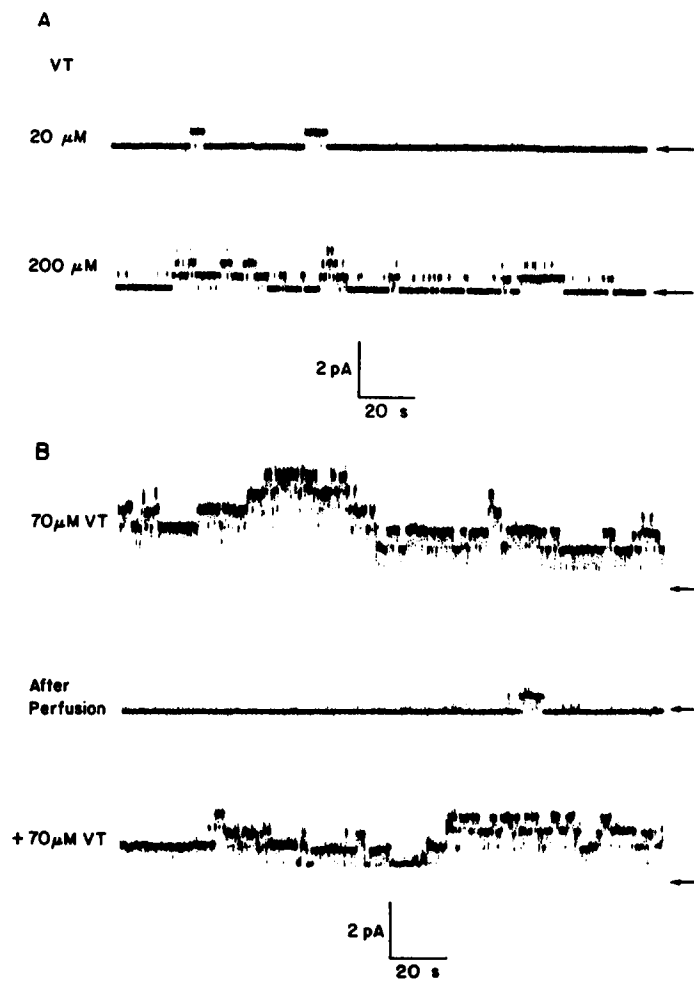
A

VT

20 μM

200 μM

2 pA

20 s

B

70 μM VT

After
Perfusion

+ 70 μM VT

2 pA

20 s

FIGURE 7.   Effect of VT concentration on channel behavior. Channels were incorporated into a bilayer in the presence of 20 μM VT, and records were collected for several minutes (A, upper trace). The VT concentration was then increased to 200 μM (A, lower trace). In a separate membrane (B), channels were observed with 70 μM VT (upper trace), and then the aqueous solutions were extensively perfused with VT-free solution containing liposomes (8 mg/ml) in order to assess the reversibility of VT activation. In spite of the removal of VT from the aqueous phase, occasional channel openings were still observed (center trace). Channels immediately appeared again after VT was added back to the inside solution (lower trace).

encountered two serious problems in our system that have made it impossible to carry out a quantitative study on the mechanism by which VT activates the Na^+ channel. First, we have never obtained membranes containing only a single channel, nor have we been able to determine the number of channels in the bilayer, since the maximal VT effect leads only to partial channel opening. Second, we have never been able to establish true equilibrium of VT in the planar bilayer; complete removal of the alkaloid from the aqueous phase by extensive perfusion with VT-free solutions resulted in only partial (70–90%) reversal of VT activation (Fig. 7B). Considering VT's lipid solubility, we perfused with VT-free solutions containing liposomes, but even this extreme maneuver did not lead to complete reversal of the alkaloid's effect. We therefore conclude that the system does not reach true equilibrium on the time scale of our experiments, and that VT binds to the Na^+ channel from the membrane, not the aqueous, phase. In spite of these problems, our single channel records are qualitatively consistent with the idea that VT activates the channel by stabilizing a long-lived open state lasting several seconds, and that the channel can enter a nonconducting state with the toxin still bound.

Comparison of Ion Conduction in Na^+ Channels Activated by VT or BTX

Symmetrical solution conductance. The initial observation (Fig. 5) that the value of open channel Na^+ current depends upon the particular alkaloid activator used to open the channel has fueled further comparison of ion conduction through the toxin-activated channels. Open channel *I-V* curves in symmetrical solutions of Li^+, Na^+, and K^+ are shown in Fig. 8. For both Na^+ and Li^+, the *I-V* curves were roughly linear, and the BTX-activated channels showed higher conductance than did VT-activated channels. In striking contrast, K^+ currents were identical for BTX and VT over the entire voltage range and showed significant inward rectification. The ion selectivity as determined by single channel conductance reveals a different selectivity sequence for each alkaloid-modified toxin. Na^+ was still the ion conducted most easily through either toxin-modified channel; Li ions passed more easily than K ions through BTX-modified channels; in VT-modified channels, however, K^+ was more conductive than Li^+.

The single channel conductance saturates with increasing concentrations of each conducting ion tested in either alkaloid-modified channel. For channels that can be occupied by, at most, a single conducting ion, the conductance (γ) interpolated to zero voltage under symmetrical ion conditions is expected to follow a rectangular hyperbola (Läuger, 1973; Hille, 1975):

$$\gamma = \gamma_{max}[X^+]/(K_D + [X^+]), \tag{1}$$

with maximum conductance γ_{max} and apparent dissociation constant K_D. Fig. 9 shows that Na^+ conductance does follow a rectangular hyperbola from 3 to 500 mM. For both VT and BTX, the half-saturation concentrations of the curves for Na^+ are similar (5.5 and 6.5 mM, respectively), but the maximum conductance is twice as high in BTX (20 pS) as in VT (10 pS). Our smaller amount of data on Li^+ and K^+ conductance does not allow calculation of half-saturation concentrations for these ions, but we see that these conductances also reach limiting
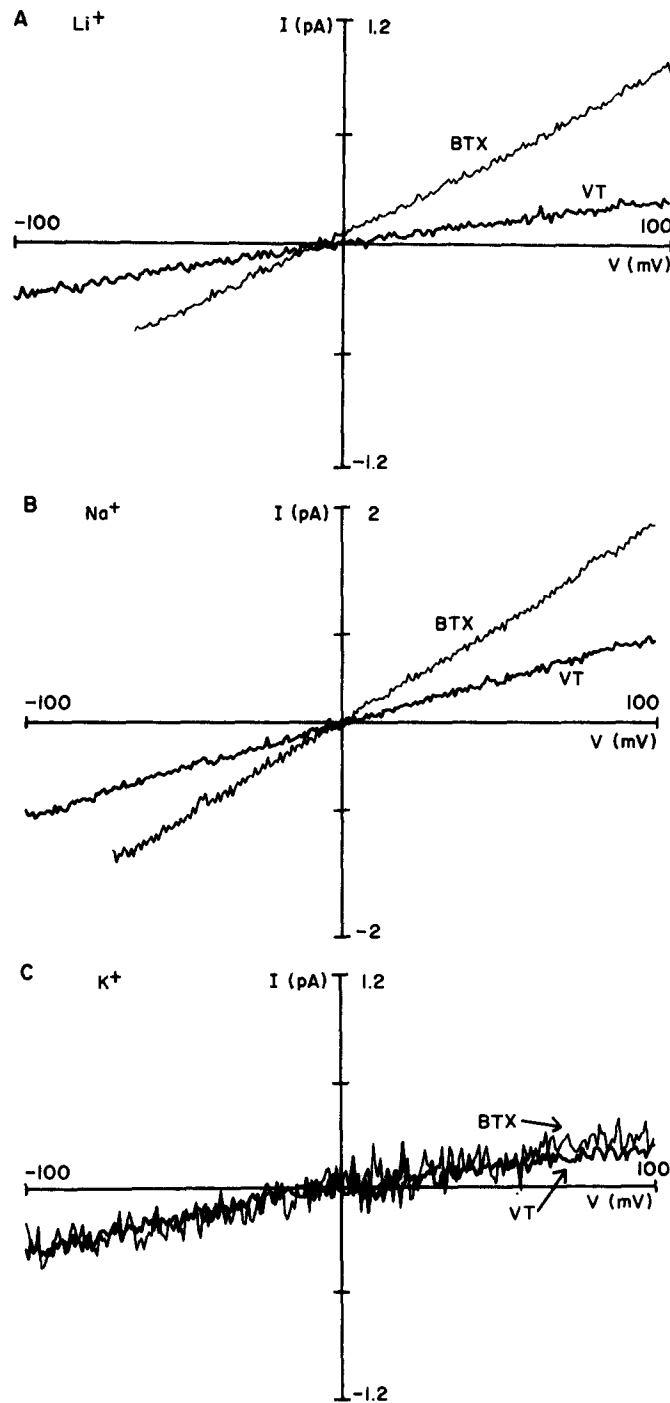
FIGURE 8.   Ionic selectivity of alkaloid-activated Na^+ channels: ionic conductance. Open channel I-V curves were measured in symmetrical solutions of 200 mM Li^+ (A), Na^+ (B), or K^+ (C), in the presence of VT (35 μM) or BTX (0.2 μM) as indicated.

values at high concentrations. Again, the absolute conductances for Na^+ and Li^+ differ with BTX and VT, but the K^+ conductances are identical for both activators, across the entire range of concentrations tested. As measured by conductance under symmetrical ionic conditions, then, the ionic selectivity of the BTX-activated channel is higher than that of the VT-activated channel. These data on symmetrical solution conductances are summarized in Table II.

Bi-ionic conditions. In establishing the system here, we showed open channel *I-V* curves (Fig. 3) under bi-ionic conditions for several ions permeating the VT-activated channel. Table I reports more extensive measurements of bi-ionic reversal potentials using all the group IA cations at several different concentra-

TABLE II

Comparison of Ion Conductance and Permeability Ratios

Toxin	Ion	γ (at 500 mM)	γ (Na^+)/ γ(ion)	$P(Na^+)/P$(ion)
VT	Na	10±1	1	1
	Li	3.6±0.5	2.9	1.1
	K	5.0±0.2*	2.1	2.9
	Rb	ND‡	—	12
	Cs	ND	—	≥20
BTX	Na	21±2	1	1
	Li	9±2	2.2	1.1
	K	5.7±0.5*	3.6	8.7
	Rb	ND	—	≥33
	Cs	ND	—	>45
Untreated	Na	—	—	1
	Li	—	—	1.1
	K	—	—	11.6
	Rb	—	—	>83
	Cs	—	—	>78

* Because the *I-V* relationship for K^+ is asymmetric (see Fig. 8 and text), the channel conductance reported here is determined over the voltage range −100 to 0 mV.
Single channel conductances, γ, were determined under symmetrical solution conditions, at 500 mM ion concentration. Permeability ratios, calculated from reversal potentials under bi-ionic conditions, are shown for comparison. Data for unmodified Na^+ channels are taken from Hille (1972).
‡ Not determined.

tions. Both BTX- and VT-activated channels show the same relative selectivity sequence, $Na^+ \sim Li^+ > K^+ > Rb^+ > Cs^+$, which is the sequence determined under mixed-ion conditions for unmodified and for alkaloid-activated Na^+ channels (Hille, 1972; Khodorov and Revenko, 1979; Naumov et al., 1979; Huang et al., 1982; Tanaka et al., 1983; Moczydlowski et al., 1984a). It is clear, however, that substantially larger reversal potentials are seen with BTX as the activator than with VT. The Na^+/K^+ reversal potential, for example, is ~55 mV with BTX (close to the value for the unmodified channel), and only ~28 mV with VT. These values of bi-ionic reversal potential remain constant over a 10-fold range of absolute ion concentration.

Block of Outward Current by Internal K$^+$

Fig. 10 shows open channel *I-V* curves in symmetrical solutions of 57 mM Na$^+$ containing symmetrical additions of K$^+$. With both VT and BTX, K$^+$ blocks Na$^+$ current in a voltage-dependent way, with outward current being blocked much
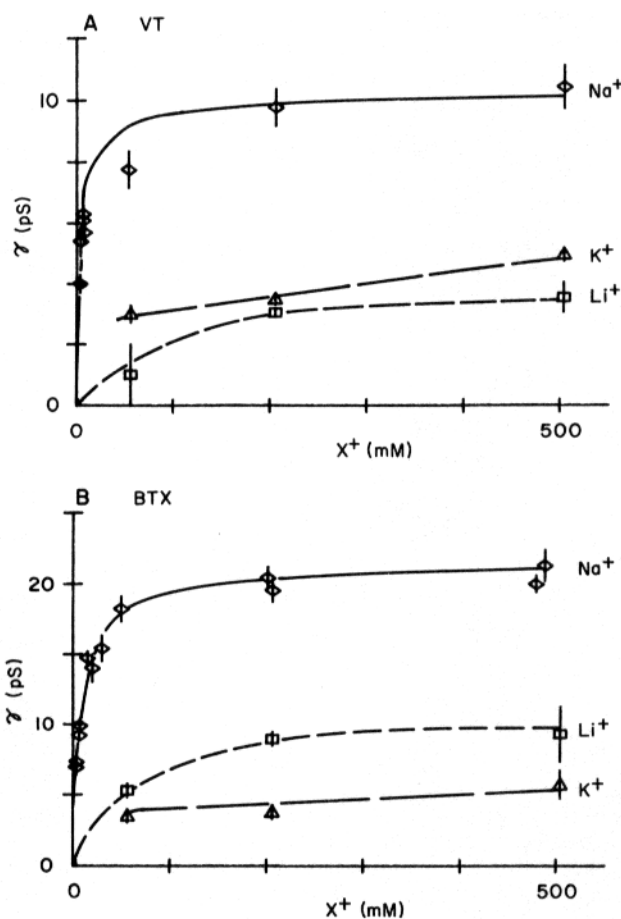
FIGURE 9. Variation in channel conductance with ion concentration. The single channel conductance (γ), interpolated to zero voltage, was measured under symmetrical solution conditions in the range −100 to 100 mV for Li$^+$, Na$^+$, and K$^+$ at the indicated concentrations. (*A*) VT-activated channels. (*B*) BTX-activated channels (note that the scales of the ordinate in *A* and *B* are different). Each point represents the mean and SEM derived from 15–100 hand measurements in three to eight bilayers. The solid curves are drawn according to rectangular hyperbolas derived from Scatchard plots. (*C*) Scatchard plots of more extensive data for Na$^+$. Least-squares regression lines are shown, with the following parameters: $K_D = 5.5 \pm 1.2$ mM for VT, and 6.5 ± 1.4 mM for BTX; $\gamma_{max} = 10.3 \pm 0.5$ pS for VT and 20 ± 0.8 pS for BTX. Errors on these parameters represent the ranges of acceptable values, and were estimated subjectively by rocking the line about the least-squares value.

more strongly than inward current. By varying the concentration of K^+, we calculate an apparent inhibition constant for K^+, and this is shown in Fig. 11. While the apparent blocking affinity of K^+ at zero voltage is not much affected by the alkaloid toxin, it is clear that the K^+ block is less voltage dependent with VT than with BTX as the channel activator. We wish to emphasize that the alkaloid-dependent differences in the voltage dependence of K^+ need not mean that the physical location of the K^+-blocking site changes with the activator; this voltage dependence can be quite complicated since K^+ binding is competitive with Na^+ and Na^+ conduction is alkaloid dependent.
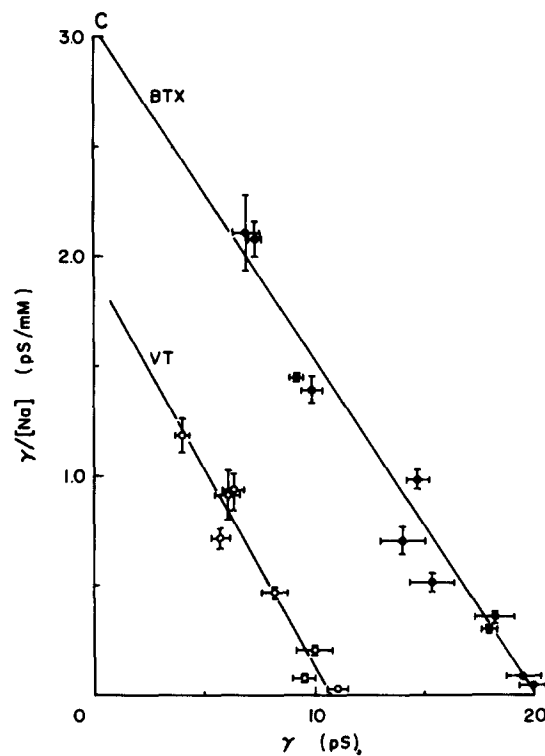

FIGURE 9C.

DISCUSSION

The alkaloid neurotoxins VT and BTX have for many years been known to elicit a persistent activation of Na^+ channels. Mechanistic pictures of the action of these toxins are still developing, and nagging questions concerning the effect of these toxins on the ionic selectivity of the Na^+ channel have remained unanswered. Our results contribute to an understanding of toxin-induced channel activation and settle several questions about the effects of the toxins on ionic selectivity. In summary, we can draw two conclusions from examining toxin-activated single Na^+ channels: (a) that present models of BTX and VT activation are consistent with the single channel characteristics observed, and (b) that the

ionic selectivity of the Na^+ channel is clearly and profoundly affected by the activator toxin used.

Before discussing our specific results here, we should address the basic question of whether the two different alkaloid neurotoxins bind to the same, but differently modified, population of Na^+ channels rather than to separate populations
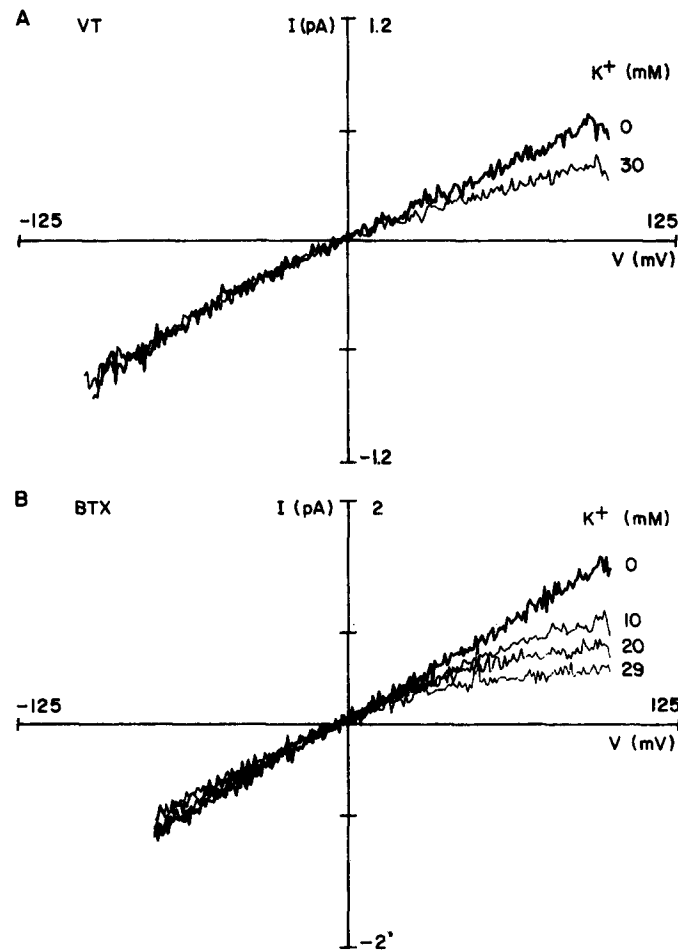

FIGURE 10.   Asymmetric block of Na^+ currents by K^+. Open channel *I-V* curves were determined in symmetrical solutions of 57 mM Na^+, to which K^+ was added symmetrically to the concentrations indicated. (A) VT-activated channels. (B) BTX-activated channels.

of Na^+ channels. Our own results do not rigorously establish that the channels observed with VT present arise from the same molecular population as those studied in the presence of BTX. We do point out, however, that the identical blocking behavior of the STX derivative C1 in both alkaloid-modified channels does suggest that the alkaloids act on a single population of Na^+ channels. This similarity is particularly pertinent in light of the well-known differences in

guanidinium toxin binding to different populations of Na^+ channels within the same tissue or organism (Moczydlowski et al., 1986).

A much stronger argument that the alkaloid neurotoxins do indeed operate on the same population of Na^+ channels can be made from biochemical studies. Catterall and colleagues (Catterall, 1975, 1977; Tamkun and Catterall, 1981) have shown through radioactive flux work that VT reduces the activation of Na^+ channels by BTX quantitatively, as expected if both activators compete for the same site on the channel. Similarly, Weigele and Barchi (1982) confirmed this conclusion through studies of purified Na^+ channels reconstituted into liposomes at an average of a single channel per liposome. In this system, both BTX and VT made the same internal space accessible to $^{22}Na^+$. Finally, Catterall et al. (1981) showed directly that the binding of a BTX analogue to Na^+ channels is competitively inhibited by VT. These results all strongly violate the hypothesis
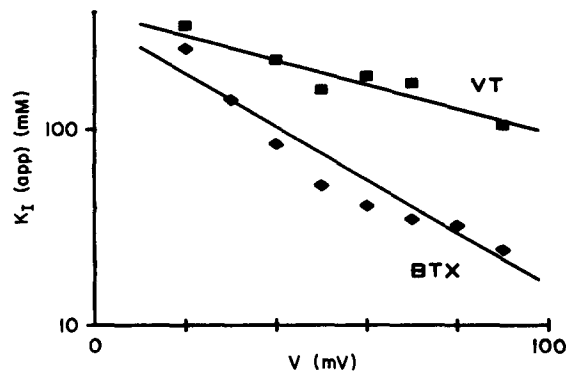

FIGURE 11. Voltage dependence of Na^+ channel block by K^+. The apparent inhibition constants of Na^+ currents by K^+ were calculated as a function of applied voltage from data as in Fig. 10, with VT (squares) or BTX (diamonds) as the activator. The data were compiled from ≥ 10 bilayers in the presence of either activator. The straight lines were the best fit of a linear regression analysis. The slopes of these lines give an e-fold increase in affinity of K^+ block per 67 mV for VT and per 31 mV for BTX. Zero-voltage inhibition constants are similar for both activators (420 mM for VT-modified and 350 mM for BTX-modified Na^+ channels).

that VT and BTX activate different populations of Na^+ channels and strongly support the idea that both toxins can act on the same population of channels. For these reasons, we consider that the differences that we observed in the behavior of alkaloid-activated channels are due to differences in the effect of the activator on the channel, not to differences in channel populations.

Mechanism of Action of Alkaloid Neurotoxins

The first proposal concerned with the mechanism of alkaloid toxins on Na^+ channels considered their properties as activators of the Na^+ channel (Catterall, 1975, 1977; Tamkun and Catterall, 1981). The toxins were shown to compete for occupancy of a specific site on the channel. When bound to this site, BTX behaves as a "full agonist," which prevents the channel from inactivating. The effect of BTX has been amply confirmed by single Na^+ channel behavior (Quandt

and Narahashi, 1982; Krueger et al., 1983; Huang et al., 1984; Moczydlowski et al., 1984a). VT has been postulated to act as a "partial agonist," allowing the VT-modified channel to close, such that its probability of being open is only ~0.6 at maximal doses of VT (Catterall, 1977). Our data palpably support this idea, since we observed opening and closing events even at high concentrations of VT (Fig. 7A). Catterall's (1977) original allosteric model of alkaloid toxin action expressed these ideas precisely:

$$
\begin{array}{ccc}
C & \longleftrightarrow & O \\
\updownarrow & & \updownarrow \\
C\text{-}A & & O\text{-}A
\end{array}
\qquad \text{(Scheme I)}
$$

where "C" represents the unliganded, nonconducting states of the channel (closed and inactivated), "O" represents the conducting states, and "A" denotes states with toxin bound. This model successfully explained the activator properties of the alkaloids at equilibrium, but Sutro (1986) and Leibowitz et al. (1986) have recently obtained electrophysiological results showing that the above model is inadequate kinetically. In particular, their voltage-clamp experiments on frog muscle imply that VT can bind to the channel only after it opens. This recent model specifies a different kinetic pathway to reach the same equilibrium states in the original scheme:

$$
\begin{array}{ccc}
C & \longleftrightarrow & O \\
& & \updownarrow \\
C\text{-}A & \longleftrightarrow & O\text{-}A
\end{array}
\qquad \text{(Scheme II)}
$$

Unfortunately, we are not able to test this model of VT action quantitatively at the single channel level using the planar bilayer system, as we cannot reliably obtain bilayers with only a single Na^+ channel. Qualitatively, however, our single channel data are inconsistent with the kinetic pathway of Scheme I, which demands that both open and closed durations of the single channel must increase without limit as the concentration of VT is increased. This clearly does not occur (Fig. 7). The data we present are consistent with Scheme II, which anticipates frequent opening and closing events even at high VT concentrations.

The dramatically different action of BTX, a "full agonist," can easily be fitted into Scheme II. In this case, the toxin-bound form of the channel closes only infrequently, and so the "C-A" form in the model is negligibly present. Since dissociation of BTX occurs on the order of hours (Catterall et al., 1981), once BTX has bound, the channel appears to be "locked" into an open state. We should emphasize that all of these models are consistent with Catterall's (1975, 1977) earlier equilibrium studies on toxin activation. The models differ only with respect to their kinetic predictions. We also emphasize that the models are incomplete in that they do not distinguish between resting and inactivated states of the channel, but lump all nonconducting states into a single "closed" state. More fully elaborated versions of these schemes have recently been proposed and tested at the whole-cell level (Sutro, 1986; Leibowitz et al., 1986).

Ionic Selectivity of Toxin-activated Na⁺ Channels

A major result of this study is the direct demonstration that the ionic conduction properties of the Na^+ channel depend strongly upon the toxin used to activate it. The notion that ionic conduction through Na^+ channels is altered by alkaloid neurotoxins has been indicated by numerous voltage-clamp and ion flux studies on whole cells and purified Na^+ channels reconstituted into liposomes (Mozhayeva et al., 1977, 1982; Khodorov, 1978; Huang et al., 1979; Khodorov and Revenko, 1979; Naumov et al., 1979; Tanaka et al., 1983; Leibowitz et al., 1986). However, these indications have been only qualitative because of the uncertainties associated with working on a population of modified channels in the presence of other conductances in the cell membrane. Our single channel measurements in reconstituted membranes provide a direct and quantitative demonstration of the differences in ion conduction properties of Na^+ channels activated by the two alkaloid neurotoxins.

The primary result is that VT-activated channels are less selective than BTX-activated channels. This is true whether selectivity is measured by single channel conductance or bi-ionic reversal potential. We cannot address questions of the unmodified Na^+ channel in this system, but we can compare our bi-ionic measurements with those made on native Na^+ channels in the cell membrane. We have found (Table I) that the reversal potentials measured for BTX-activated channels in bilayers are similar to those determined on unmodified channels of frog node of Ranvier (Hille, 1972). The selectivity for Na^+ over K^+, for example, is <3-fold for VT-activated channels, but is 9–12-fold for both BTX-activated and unmodified channels, respectively.

The absolute single channel conductances under symmetrical solution conditions are also different for VT and BTX, with BTX-modified channels generally yielding larger single channel conductances. In pure Na^+ solutions at physiological ionic strength, for example, channel conductance is about twice as large with BTX (20 pS) than with VT (9 pS). Furthermore, since the apparent dissociation constants for Na^+ are similar in BTX and VT, we imagine that the alkaloids do not affect the binding sites for Na^+ within the permeation pathway. Therefore, we propose that the toxins mainly affect the unfavorable transition-state energies experienced by Na^+ as it permeates the channel. These transition-state energies would be higher in the presence of VT than with BTX. The same conclusion holds for Li^+, which shows a substantially lower conductance than does Na^+, but no difference in permeability as measured by bi-ionic reversal potential.

To understand the effects of these activators on the conduction process, we begin with the simplest proposal: that the effects of the alkaloids are expressed at a particular place along the conduction pore, and that this structural change is independent of the permeating ion. Each ion traversing the Na^+ channel experiences a "free-energy profile," which is a reflection of the channel's structure (Hille, 1975). The differences in ion selectivity observed here can only mean that the physical structure of the permeation pathway of the Na^+ channel differs for the different alkaloid activators. We envision these structural differences as being quite small and subtle, since even small alterations (<0.1-nm movements)

in an ion-liganding region could be expected to elicit large differences in interaction energy (Armstrong, 1975; Hille, 1975).

The differences in cation permeation between VT and BTX are not attributable to differences in cation binding (at least for Na^+; Fig. 9). Therefore, it is reasonable to postulate that the alkaloid activators mainly affect an energy peak, or transition state, along the permeation profile. This proposal is consistent with the fact that the bi-ionic reversal potentials, which are sensitive to peak energies, are strongly affected by the alkaloid toxins.

In the face of these differences in ionic selectivity, it is intriguing that in pure K^+, the conduction process appears to be identical for both alkaloids. Currents with BTX and VT are the same at all voltages and K^+ concentrations, and the inward rectification, unique to K^+ here, is present with both alkaloids. This inward rectification, and the asymmetrical blocking of Na^+ currents by K^+, could be due to a K^+-binding site positioned asymmetrically within the channel, lying closer to the internal side; a very similar situation has been studied in detail for Cs^+ conduction and block in a K^+ channel from sarcoplasmic reticulum (Cukierman et al., 1985).

These similarities in K^+ conduction may be merely coincidental, but we prefer to speculate that they carry meaning: that the rate-limiting barrier for K^+ is located at a different place in the channel than those for Na^+ and Li^+, a place that is not structurally sensitive to the alkaloid activator. The most fully developed models of Na^+ channel conduction (Hille, 1971, 1972, 1975) place the rate-limiting step for poorly permeating species like K^+ at a "selectivity filter," a liganding structure that is the narrowest part of the conduction pore. Ions like K, which cannot be easily dehydrated and re-solvated by groups in this structure, will be discouraged from traversing the selectivity filter.

Most views of the Na^+ channel have tacitly assumed that this selectivity structure also represents the rate-limiting step for Na^+. Our results, however, argue that readily permeating ions are not rate-limited at the selectivity filter at all, and that these ions can easily negotiate this structure while experiencing higher permeation barriers elsewhere in the pore. It is this Na^+-limiting region of the conduction pathway that apparently takes on slightly different structures with the different activators. This hypothesis can be investigated further by studying conduction by other cations, such as Rb^+ and Cs^+, which, like K^+, permeate poorly.

With these differences in ionic selectivity in mind, we note that the interaction of the guanidinium toxin C1 with the channel is totally unaffected by the alkaloid activator. The rates of C1 association and dissociation are identical with BTX and with VT, as is the voltage dependence of these rates. This result further suggests that the effects of the alkaloid toxins on the Na^+ channel's open pore structure are highly localized at one particular region within the conduction pathway.

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