Kinetics of Veratridine Action on Na Channels of Skeletal Muscle

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ABSTRACT Veratridine bath-applied to frog muscle makes inactivation of I_{Na} incomplete during a depolarizing voltage-clamp pulse and leads to a persistent veratridine-induced Na tail current. During repetitive depolarizations, the size of successive tail currents grows to a plateau and then gradually decreases. When pulsing is stopped, the tail current declines to zero with a time constant of ~3 s. Higher rates of stimulation result in a faster build-up of the tail current and a larger maximum value. I propose that veratridine binds only to open channels and, when bound, prevents normal fast inactivation and rapid shutting of the channel on return to rest. Veratridine-modified channels are also subject to a "slow" inactivation during long depolarizations or extended pulse trains. At rest, veratridine unbinds with a time constant of ~3 s. Three tests confirm these hypotheses: (*a*) the time course of the development of veratridine-induced tail currents parallels a running time integral of g_{Na} during the pulse; (*b*) inactivating prepulses reduce the ability to evoke tails, and the voltage dependence of this reduction parallels the voltage dependence of h_∞; (*c*) chloramine-T, *N*-bromoacetamide, and scorpion toxin, agents that decrease inactivation in Na channels, each greatly enhance the tail currents and alter the time course of the appearance of the tails as predicted by the hypothesis. Veratridine-modified channels shut during hyperpolarizations from −90 mV and reopen on repolarization to −90 mV, a process that resembles normal activation gating. Veratridine appears to bind more rapidly during larger depolarizations.

INTRODUCTION

Among the powerful tools we have for studying Na channels are toxins and pharmacological agents that modify channel function. The alkaloid neurotoxins batrachotoxin (BTX), aconitine, and grayanotoxin all have similar effects on Na channels (Khodorov et al., 1975; Khodorov, 1978; Khodorov and Revenko, 1979; Herzog et al., 1964; Schmidt and Schmitt, 1974; Mozhayeva et al., 1976, 1981; Campbell, 1982; Seyama and Narahashi, 1981; reviewed by Catterall, 1980). They bind to Na channels and remove normal fast inactivation. Modified channels also remain open at the normal resting potential, where unmodified channels are shut. Hyperpolarizations on the order of 50 mV from the normal

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resting potential will shut modified channels and depolarizations back to rest will reopen them. This shutting and reopening resembles normal activation gating, which is shifted to more negative voltages and has modified kinetics.

Veratridine has been placed in the same functional class as the other alkaloid toxins on the basis of pharmacological studies (Catterall, 1975*a*, *b*, 1977*b*). These agents stimulate the uptake of radioactive Na ions into mouse neuroblastoma cells by different amounts, depending on the toxin used. Applying an excess of a less potent toxin in conjunction with a more potent toxin decreases the effect of the more potent toxin in a manner consistent with competition for the same site (Catterall, 1975*a*, *b*, 1977*b*). Since veratridine has pharmacological effects similar to those of BTX, grayanotoxin, and aconitine, and competes with them for binding, the electrophysiological effects should also be similar.

Previous electrophysiological experiments have indicated that veratridine's effects differ significantly from those of the other alkaloid neurotoxins. Ulbricht (1969, 1972*a*, *b*) and Leicht et al. (1971*a*, *b*) found an Na current in veratridine-treated fibers that developed with a time course of seconds over a voltage range similar to the voltage range for normal Na channel activation. This current did not display fast inactivation, but it did inactivate partially in tens of seconds. Upon return to the normal resting potential, the slow current decayed to zero over a few seconds. The development and disappearance of this current was interpreted as the opening and shutting of modified Na channels whose gating kinetics were several orders of magnitude slower than normal channels. This contrasts with Na channels modified by the other alkaloid neurotoxins, which open and shut in milliseconds at potentials hyperpolarized by ~50 mV from rest. One of the motivations for my study of veratridine was to understand why veratridine and the other alkaloid neurotoxins seem so similar in pharmacological flux assays but so different in voltage-clamp work.

Several investigators (Catterall, 1975*b*, 1977*b*; Stallcup, 1977; Jacques et al., 1978; Kreuger and Blaustein, 1980; Tamkun and Catterall, 1981) have found that when scorpion toxin is applied in conjunction with any of the alkaloid neurotoxins to cells or vesicles containing Na channels, the Na influx is enhanced compared with the Na influx when the alkaloid neurotoxin is applied alone. Veratridine and BTX also enhance the binding of scorpion toxin to Na channels (Catterall, 1977*a*; Tamkun and Catterall, 1981), although the effect is less pronounced than the enhancement of alkaloid toxin effects by scorpion toxin. These studies were done using Na flux assays, scorpion toxin binding assays, and voltage-sensitive dyes. A second goal of my studies was to use electrophysiological techniques to further examine the interactions between these toxins. Preliminary reports have been made to the Biophysical Society (Sutro, 1984*b*; Leibowitz et al., 1985).

METHODS

Single muscle fibers were dissected and voltage-clamped by the method of Hille and Campbell (1976), with these small modifications: The transmembrane currents were obtained directly as the voltage drop across a 200-kΩ resistor. To protect against high-frequency oscillations from the series resistance compensation, a high-frequency detector was included that shut off the clamp if such oscillations occurred. Finally, the command

voltage pulse was used as the voltage signal to the leak compensator, since it was more consistent than the membrane voltage signal used in the original design.

During an experiment, twitch fibers were removed from the semitendinosus muscle of *Rana pipiens* in a standard Ringer solution of 115 mM NaCl, 2 mM $CaCl_2$, 2.5 mM KCl, and 4 mM morpholinopropane sulfonic acid (MOPS) buffer at pH 7.4. The fibers were placed immediately in the plastic chamber, which was flooded with an "internal" solution containing either 115 mM CsF and 5 mM NaF or (where noted) 120 mM CsF. Once the fiber was mounted and vaseline seals were established, the ends were recut and the solution level was lowered to create four separate pools. The chamber was then moved to the voltage-clamp apparatus and the solution in the test pool was exchanged for Ringer solution. Control currents were obtained and the "external" Ringer was exchanged for a test solution of R̃inger plus the drug being investigated.

Circulating coolant at a temperature of 7°C kept the chamber near 9°C, except where otherwise noted. The holding potential was set at the "normal resting potential" of −90 mV (inside potential referenced to outside potential), except where noted. Membrane current and voltage were displayed on a storage oscilloscope and photographed for future analysis. The current was usually filtered by an 11-kHz low-pass filter. The data displayed in Fig. 9 were obtained using the apparatus described in the following paper (Leibowitz et al., 1986).

Veratridine (Aldrich Chemical Co., Milwaukee, WI) was applied by washing the 0.15-ml test pool with either 2 or 5 ml of Ringer solution plus veratridine (hereafter referred to as veratridine Ringer). Veratridine effects persist after washing with normal Ringer, presumably because veratridine partitions preferentially into the membrane (Balerna et al., 1975). Repeated washings result in a partial washout of veratridine, so experiments were normally done in an external solution containing veratridine. This is in agreement with the finding of Ulbricht (1972*b*) that veratridine can be completely washed out after applications lasting <1 min, but not after prolonged applications, and the finding of McKinney (1984) that veratridine washes out with a half-time of 25–30 min.

Many of my experiments investigated the combined effect of veratridine and a purified component of *Leiurus quinquestriatus* scorpion venom (Catterall, 1976), kindly provided by Dr. W. A. Catterall (University of Washington, Seattle, WA). In my experience, solutions containing both veratridine and scorpion toxin rapidly damaged the fiber. However, since the effects of the scorpion toxin are essentially irreversible, I was able to wash the solution containing scorpion toxin out of the test pool and then return to a test pool solution of veratridine Ringer.

The usual procedure was to measure control currents in an external solution of Ringer and then change the external solution to veratridine Ringer, where another set of currents was measured. Then the test pool was flushed with Ringer, followed by a wash with ~2 ml of Ringer plus scorpion toxin and ~0.1% bovine serum albumin (hereafter referred to as scorpion toxin Ringer). The Na currents were then monitored to determine when the alteration of the Na currents by scorpion toxin was complete—a few minutes. Usually a final wash of 5 ml of Ringer was done before changing the external solution to veratridine Ringer. With this procedure, the fiber often survived long enough for me to make the required measurements. A similar procedure was used in experiments on the effects of either *N*-bromoacetamide (NBA) or chloramine-T (both from Sigma Chemical Co., St. Louis, MO) in conjunction with veratridine.

In some instances, Na currents have been converted to Na permeabilities by use of the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949):

$$I_{Na} = P_{Na}[Na]_o \frac{F^2E}{RT} \frac{\exp[(E - E_{Na})F/RT] - 1}{\exp(EF/RT) - 1},$$

where E and E_{Na} represent the membrane potential and the Na reversal potential (determined as the value of E_{Na} that gave the smoothest Na permeability vs. voltage curve). Analysis was carried out using the LM[2] computer developed by Dr. T. H. Kehl's group at the University of Washington (Kehl et al., 1975; Kehl and Dunkel, 1976).

RESULTS

Initial Observations

Fig. 1 shows the Na current from a voltage-clamped single frog skeletal muscle fiber during a depolarizing pulse, before and after the external application of 100 μM veratridine. In the untreated fiber, a depolarization causes Na channels to activate, and a normal, transient inward Na current appears, rising to a

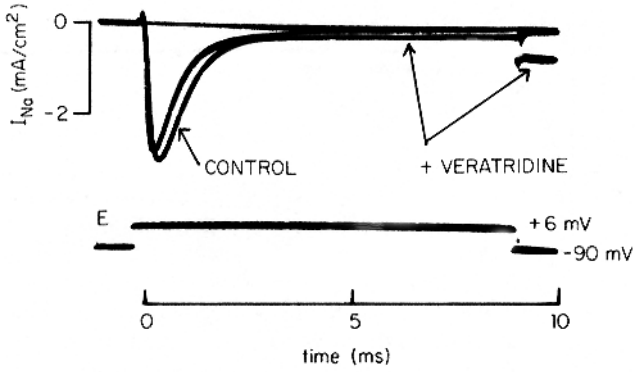


FIGURE 1. Veratridine-modified fibers display persistent Na tail currents after depolarizing pulses. Na currents measured during a 9-ms depolarizing pulse from a holding potential of −90 mV to a test potential of +6 mV are shown. The control current was measured with the fiber bathed in standard Ringer solution with the circulating coolant at 7°C. Then the bathing solution was changed to 100 μM veratridine Ringer, and current was measured again. Data were obtained using a voltage clamp with separate electrodes for measuring voltage and passing current.

maximum and then decreasing as Na channels inactivate. By the end of the pulse, inactivation is complete and Na current is near zero. Upon the return to the resting potential, there is a brief tail of capacitive current and nearly no residual ionic current. Treatment with veratridine has two obvious effects: (*a*) inactivation fails to go to completion during the test pulse in the veratridine-treated fiber, and (*b*) a persistent "tail" of Na current is seen after the return to the holding potential. Much of this paper is a description of this special veratridine-induced tail current. (The "hook" seen early in the tail reflects an imperfect subtraction of capacitive current and is frequently not present [e.g., Fig. 6].)

When a veratridine-treated fiber is subjected to a series of short (9 ms) depolarizing pulses at a rate of one every 110 ms (Fig. 2), the tail current increases toward a maximum with each successive pulse. At the same time, the noninactivating current during the pulse increases with each pulse and the transient peak current decreases. Hence, as Ulbricht (1965, 1969) observed, the induction of the tail current and the noninactivating current is cumulative with

repetitive stimulation and occurs at the expense of the transient component of Na current. A quantitative analysis of the induction of tail current is given in this paper, and a description of the loss of peak current is given in the following paper (Leibowitz et al., 1986).

A working hypothesis explaining the tail current and the noninactivating current is that veratridine binds poorly to a shut Na channel and binds much better to an open Na channel. Once bound, veratridine holds the channel in the open position, so that it neither inactivates during the depolarizing test pulse nor shuts upon return to rest. This hypothesis was originally proposed by Hille (1968) for bis(chlorophenyl)trichloroethane (DDT) and veratridine. If veratridine bind-

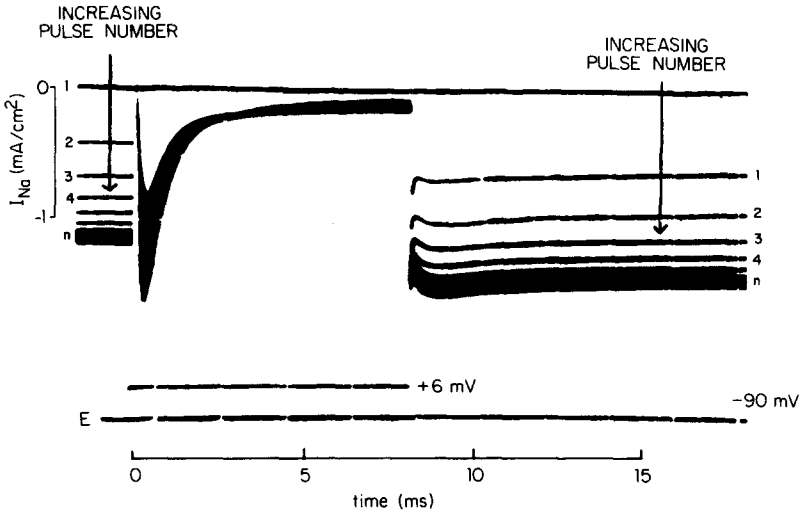
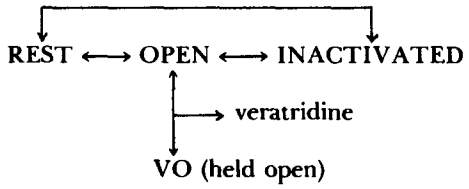


FIGURE 2. Repeated stimulation modifies Na channels. Same fiber as in Fig. 1. Depolarizing pulses from −90 to +6 mV were applied at a frequency of 1.89 Hz. The pulses were of 9 ms duration. The trace corresponding to the first sweep is numbered "1," that corresponding to the second sweep is numbered "2," and so forth.

ing to an open channel is slow compared with the length of time that the channel is open, then only a small fraction of the available channels will be modified during a single depolarizing pulse. At the resting potential, veratridine unbinds. If the unbinding is slow compared with the time between pulses, the fraction of channels with veratridine bound will increase with each pulse until the unbinding between depolarizations equals the binding during the pulse. Using a simplified state diagram of the Na channel, this can be depicted as:



In this hypothesis, binding is equated with the induction of the pharmacological effect, namely the persistent opening of channels at the normal resting potential.

Tests of the Hypothesis

Some of the kinetic predictions of the hypothesis can be tested. First, consider the rate of unbinding of veratridine. Fig. 3 shows the effect of repeated pulsing in veratridine. The time scale in this trace is so slow that the current flowing during each test pulse is too brief to photograph and only the current flowing between pulses (the tail current at the resting potential) is seen. On the voltage trace, the individual pulses also blend together, so that what is actually a series

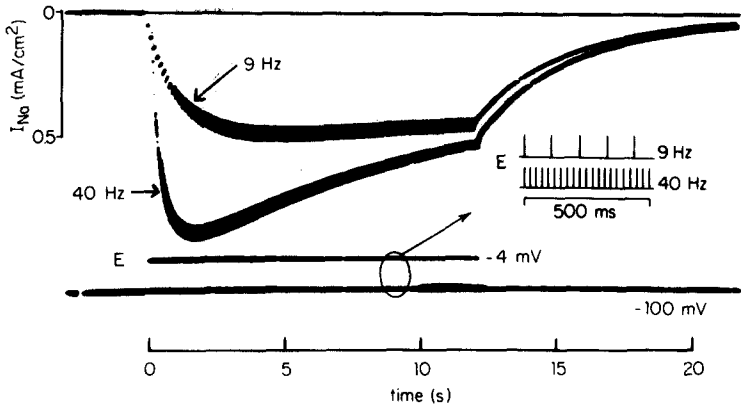


FIGURE 3. Faster repetitive stimulation modifies more Na channels. The fiber was equilibrated in 50 μM veratridine Ringer and stimulated at either 9 or 40 Hz. The time scale is so slow that the traces show 108 or 480 pulses, respectively, in a single sweep. The current flowing during the depolarizing pulses is too fast to be photographed on this time scale. What can be see are the tail currents following the depolarizing pulses. Similarly, what looks like a solid line at −4 mV on the voltage scale is actually the blending together of numerous individual pulses to −4 mV. The holding potential was −100 mV. The temperature of circulating coolant was 11°C and the B, C, and E pools contained 120 mM CsF.

of pulses appears to be a pair of continuous traces marking the period of stimulation. After the end of pulsing, the fiber is held at −90 mV, and the tail current returns to baseline with a time constant of 3.2 s. The same pulse pattern can be repeated numerous times, with similar results. In the above hypothesis, this slow decay results from the slow unbinding of veratridine from the Na channel, which permits the channel to return to the shut REST state. The long lifetime of the veratridine-channel complex, ~3 s, supports the assumption that the rate of unbinding is slow compared with the interval between pulses.

If veratridine binding requires the open state of the channel, any experimental manipulation that increases the time spent in the open state should increase the number of channels becoming modified. There should be a precursor-product relation between open channels and modified ones. One test of this is to increase the rate of pulsing (Fig. 3). At the higher pulsing rate, the veratridine-induced

current reaches a larger value than at the lower pulsing rate, while the decay of this current after the end of pulsing is unchanged. With prolonged high-frequency pulsing, the veratridine-induced tail is eventually reduced again, a slow inactivation process that is described later.

If we can equate binding with the induction of persistent current, then apparently veratridine does not bind to resting channels, since it does not cause a consistent increase in the resting "leakage" current at −90 mV. However, these observations do not rule out an alternative hypothesis that veratridine binding depends on membrane potential, rather than on the state of the channel. To test whether veratridine will bind to inactivated channels during a depolarization, I varied the length of the depolarizing pulse and compared the time course of the development of the veratridine-induced tail current with the time course of the Na current. Such experiments (Fig. 4) show that the time course of the appearance of the veratridine-induced tail current closely parallels a running time integral of Na current during the pulse. The Na current is used here as a measure of the number of open channels, and the running integral is used as a measure of the total availability of open channels for reaction with veratridine during the specified interval.

Fig. 4 shows three experiments using depolarizations to −34, +6, and +78 mV (where Na current is outward). At each potential, the time course of development of tail currents is close to the time course of the integral of Na current, even though the kinetics of the Na current are distinctly different. Hence, the rate of veratridine binding is nearly proportional to the instantaneous number of open channels. Analysis of tail currents after pulses of >3 ms duration indicates that the rate of binding to inactivated channels is ≤1.5% of the rate of binding to open channels. Although it is not obvious on the scale of Fig. 4, the initial rise of the veratridine-induced tail current slightly precedes the opening of the Na channels (see Discussion).

Another test of whether veratridine binds to inactivated channels is to measure the effect of inactivating prepulses on veratridine binding during a subsequent test pulse. Fig. 5 compares the effects of prepulses on peak I_{Na} with the effects of the same prepulses on the veratridine-induced tail currents. The curves fall closely together, which demonstrates again the ability of inactivation to prevent veratridine binding. Veratridine-induced tail currents fail to become zero at the depolarizing prepulses where inactivation is complete, probably because veratridine binds to Na channels that open during the prepulse.

Slowing Inactivation Increases Veratridine Binding

One prediction of the working hypothesis is that, since veratridine does not bind to inactivated channels, anything that removes or slows inactivation should increase veratridine binding. I have confirmed the prediction with three such substances, NBA, scorpion toxin, and chloramine-T. Fig. 6 compares I_{Na} in a fiber treated with veratridine alone to that after treatment with veratridine and NBA together. The trace taken after treatment with NBA is scaled up to make the peak closer to that taken in veratridine alone. As shown by previous work (Oxford et al., 1978; Nonner et al., 1980; Oxford, 1981), NBA-treated Na channels inactivate little during a 9-ms test pulse.

Additionally, as shown in Fig. 6, the tail current now has two parts, a fast component reflecting the shutting of NBA-modified channels without veratridine, and a greatly increased slow component reflecting a large number of veratridine-modified channels. Single channel studies (Patlak and Horn, 1982;

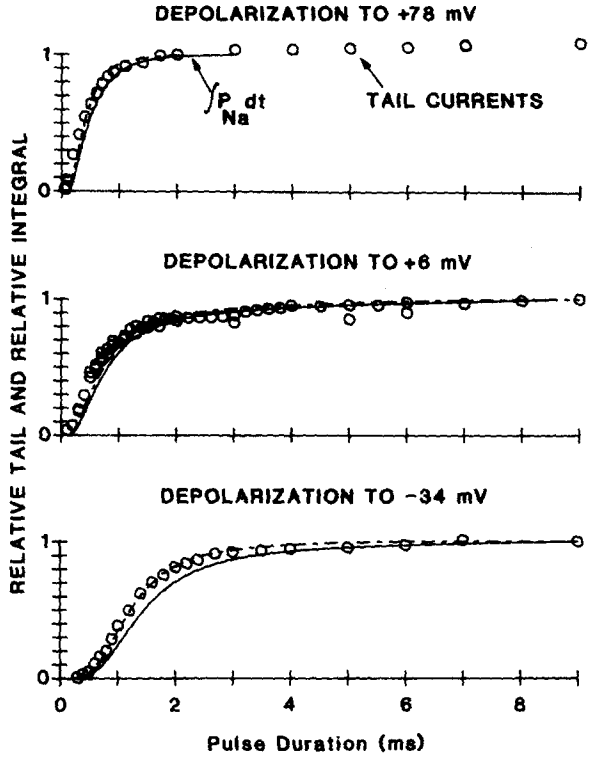


FIGURE 4. Comparison of Na tail currents with the integral of Na current flowing during depolarizing pulses. The tail currents were measured in 100 μM veratridine Ringer after individual pulses of the indicated duration. In each experiment, two 9-ms depolarizing pulses were integrated, one measured before measuring the tail currents (dashed line) and one measured after measuring the tail currents (solid line). For the depolarizations to −34 and +6 mV, both the tail currents and the integrals were normalized to be equal to 1.0 at $t = 9$ ms. For the depolarization to +78 mV, the tail currents and the integral measured before measuring the tail currents were normalized at $t = 2$ ms, and the other integral was normalized at $t = 3$ ms. The holding potential was −90 mV in all cases. Data for the depolarizations to −34 and +78 mV came from the same fiber. Tail currents from this fiber were measured 10 ms after the end of the test pulse. Tail currents from the other experiment were measured from 1 to 10 ms after the end of the test pulse.

Horn et al., 1984) have shown that the slowing of inactivation by NBA is due to channels remaining open longer, although their conductance is not altered. Hence, holding channels open longer does in fact increase the veratridine-induced tail current.

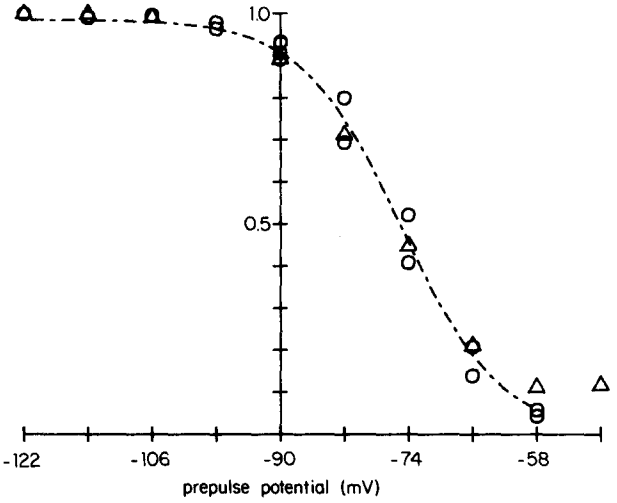


FIGURE 5. Inactivating prepulses prevent veratridine binding. These data were obtained in 100 μM veratridine Ringer using a 72-ms prepulse from a holding potential of −90 mV to the prepulse potential indicated. This was followed by a 9-ms test pulse to −18 mV, and then by a return to the holding potential. The circles represent the normalized peak current measured during the test pulse (h_∞) and the triangles represent the normalized tail current measured 10 ms after the end of the test pulse. Two h_∞ curves were measured, one just before the tail currents were measured and one just after. These were averaged, and the dashed line is a least-squares fit of a Boltzmann distribution to this average.

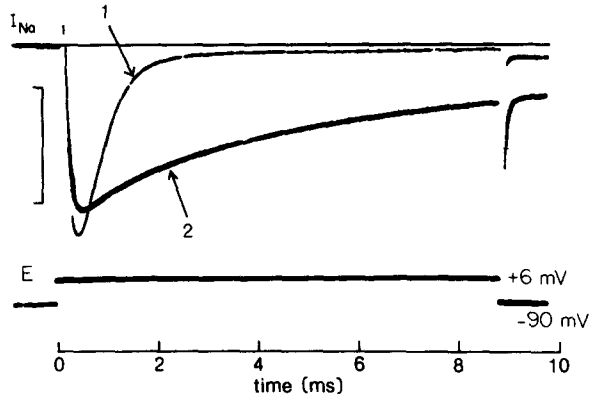


FIGURE 6. Comparison of current through Na channels modified by veratridine alone and by veratridine plus NBA. The fiber was first treated with 25 μM veratridine Ringer, and the first trace (1) was taken. The fiber was then bathed in 25 μM veratridine Ringer with 1 mM NBA added, and the second trace (2) was elicited. Test pulses were from a holding potential of −90 mV to a test potential of +6 mV and were 9 ms long. The I_{Na} scale bar represents 1.0 mA/cm^2 for the first trace and 0.5 mA/cm^2 for the second trace.

Scorpion toxins and chloramine-T also slow Na channel inactivation in frog skeletal muscle, as previously observed in other preparations (see review by Catterall, 1980; Wang and Strichartz, 1983). The application of either 50 nM *Leiurus* scorpion toxin (six experiments) or 400 μM chloramine-T (four experiments) in combination with veratridine results in proportionately larger tail currents than those seen after treatment with veratridine alone. One preliminary experiment using low internal pH to slow inactivation gave similar results.

To show that veratridine binds to channels whose inactivation is slowed by NBA, I have plotted the tail current amplitudes after pulses of different lengths, together with a running integral of the Na currents during a pulse (Fig. 7). The inactivation of channels treated with NBA and veratridine is incomplete even at pulse lengths as long as 250 ms, and the number of veratridine-modified channels increases throughout this time. The first 50 ms of a pulse is represented in Fig. 7. The agreement between the two curves is good up to ~15 ms, but then they begin to diverge. The divergence is greater at pulse lengths beyond 50 ms, and the scatter in the veratridine-induced tail currents becomes large, but the tail currents and integrals continue to grow as the pulse length increases. As with veratridine alone, the development of veratridine-induced tail currents initially appears to precede the opening of Na channels (see Discussion). Analogous experiments using either chloramine-T (one experiment) or scorpion toxin (two experiments) in combination with veratridine gave similar results.

An explanation for the divergence is that at long times a significant fraction of the open Na channels have already been modified and are not free to bind more veratridine. Since current through already modified channels is included in the running integral, the rate of continuing modification is overestimated. To account for this error, I first estimated the effective rate constant for veratridine binding to open channels (Table I). This is the ratio of the Na chord conductance early in the tail after a single depolarizing pulse, divided by the time integral of the Na chord conductance during the pulse. Assuming that this rate of binding to open channels is constant throughout the pulse, I could estimate the fraction of channels that are already modified at each time during the pulse. Then I could calculate a "corrected" time integral that does not include current through veratridine-modified channels. The results from fiber NBA1 are shown in Fig. 7. As is also true of fiber NBA2, the correction brings the Na current integral closely into line with the tail currents. For the fibers modified with chloramine-T or *Leiurus* scorpion toxin, the correction reduces, but does not completely eliminate, the discrepancy between the Na current integral and the tail currents. The remaining discrepancy, however, is small enough to be accounted for by the variation in the calculated values of the effective binding rate constant, which can be quite large, even during a single experiment [compare the values for "Veratridine plus drug (early)" and "Veratridine plus drug (later)" in Table I].

Is the entire increase in the veratridine-induced tail currents after treatment with inactivation-removing agents due to the longer open time of the channels, or is the rate of binding of veratridine to open Na channels also altered? The first two columns of effective binding rate constants shown in Table I address this question. They were calculated from Na currents taken just before and

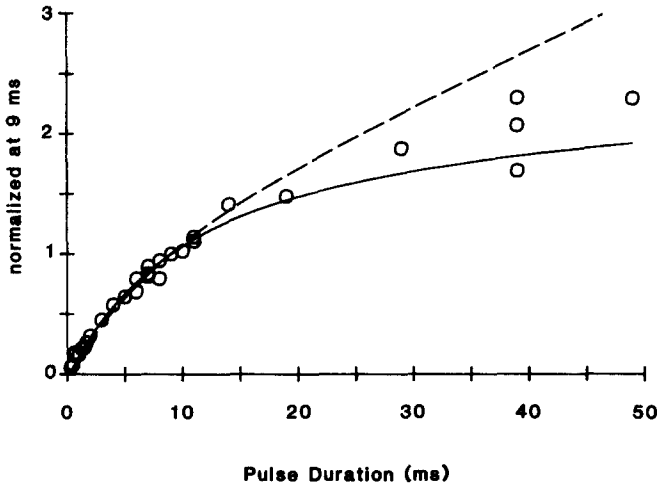


FIGURE 7. Comparison of Na tail currents and the integral of Na current in a fiber treated with veratridine and NBA in combination. The fiber was treated with 25 μM veratridine Ringer with 1 mM NBA added. The tail currents were measured 2 ms after the end of depolarizing pulses from −90 to +6 mV lasting the indicated duration. Tail currents (circles) and integrals (dashed line) were normalized to be equal to 1.0 at t = 9 ms. The corrected integral (solid line) was calculated as explained in the text.

TABLE I

Rates of Veratridine Binding to Na Channels Treated with Inactivation-removing Drugs

Fiber‡	Effective binding rate constant*		
	Veratridine alone	Veratridine plus drug (early)	Veratridine plus drug (later)§
	ms^{-1}	ms^{-1}	ms^{-1}
NBA1	0.0314	0.0297	0.022
NBA2	0.0137	0.0130	0.018
ScTX1	0.0248	0.0244	0.012
CT1	0.0194	0.0339	0.010

* The "effective rate constant" is actually a conductance ratio (see text) and would equal the true rate constant only if the conductances of normal and modified channels were equal (see Leibowitz et al., 1986).

‡ All fibers were treated with 25 μM veratridine initially. Subsequently, the fibers were treated with either 1 mM NBA (NBA1 and NBA2) or 50 nM *Leiurus* toxin (ScTX1) or 0.4 mM chloramine-T (CT1) in conjunction with veratridine (see Methods).

§ These values were determined from Na current traces recorded later in the experiment (while I was recording the tail currents at different pulse lengths) and were used to calculate current through modified Na channels at different times during the pulse. The "corrected" current values were then used to calculate the "corrected" Na current integral.

shortly after the inactivation-removing agent was applied. There was essentially no change in the effective binding rate constant after applications of NBA or *Leiurus* toxin. After the application of chloramine-T, there was an increase in the effective binding rate constant. However, this change was smaller than the variation between effective binding rate constants calculated from measurements taken at different times after modification by chloramine-T. Therefore, I conclude that neither NBA nor *Leiurus* toxin alters the rate of veratridine binding to open Na channels, and that the same may be true for chloramine-T.

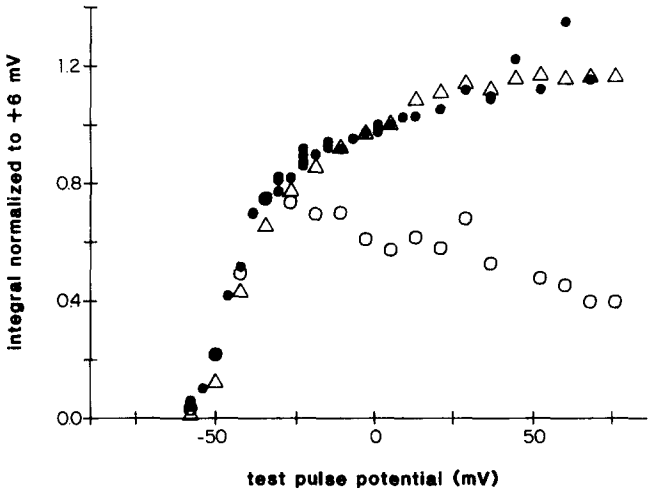


FIGURE 8. Voltage dependence of Na tail currents compared with that of the integral of Na permeability during a depolarizing pulse. The fiber was bathed in 100 μM veratridine Ringer and 8-ms depolarizations from −90 mV to the indicated test potential were applied. First, the peak Na permeabilities (triangles) were obtained. The same traces were used to obtain the integrals of Na permeability (open circles). Na tail currents (solid circles) were measured after the end of depolarizing pulses to the indicated voltages. The tail currents and peak permeabilities were normalized to 1.0 for a depolarization to +6 mV. The integrals of Na permeability are normalized to be equal to the normalized Na tail current for a depolarization to −34 mV.

Voltage Dependence of Veratridine Binding

So far, we can conclude that veratridine binding is not due solely to the depolarizing pulse, but that open channels are also necessary. However, it is possible that veratridine binding to the open channel is also voltage dependent. The veratridine-induced tail currents following single pulses to different voltages are plotted vs. membrane voltage in Fig. 8. Also shown are the peak permeability vs. voltage and the time integral of the Na permeability vs. voltage.

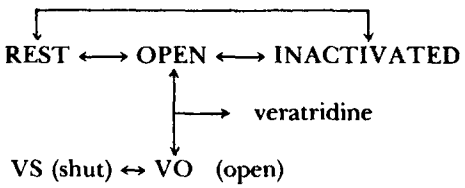
Fig. 8 shows that the time integral of Na permeability increases for depolarizations up to around −35 mV and then decreases again with larger depolarizations, which is simlar to observations in crayfish giant axon (Bean, 1981). If the binding

of veratridine depends only on the state of the channel, and if veratridine binds only to the open state, then we would expect veratridine-induced tail currents to be directly proportional to the availability of open channels as measured by the time integrals of the Na permeability. The curve of tail current vs. voltage is similar to that for the integral of Na permeability at small depolarizations, but at large depolarizations, the veratridine-induced tail currents continue to increase, while the integral of Na permeability decreases. Apparently depolarizations above about −35 mV increase the effective rate constant of veratridine binding. The good fit between the curves at potentials more negative than −35 mV and the lack of detectable veratridine-induced currents at potentials where no Na channels open are further evidence that veratridine does not bind to channels in the resting state.

Hyperpolarization Shuts Channels Modified by Veratridine

As shown above, repeated pulsing causes progressively more channels to enter the VO state and remain open at the normal resting potential of −90 mV. I now show that even veratridine-modified Na channels have voltage-dependent gating properties. In the experiment of Fig. 9, a series of depolarizing pulses was first applied to the fiber to induce a large tail current. Then a hyperpolarizing pulse to −170 mV was applied and the current was decreased. Upon the return to −90 mV, the current grew back toward its previous value. Hence, modified channels shut reversibly during large hyperpolarizations. In this experiment, the time constant of the early part of the closing was 0.4 ms and the time constant of the opening was 3.0 ms. I feel that this shutting is not due to an unbinding of veratridine because: (*a*) channels reopen upon returning to the resting potential; (*b*) shutting and reopening of the channels occurs with a time course at least two orders of magnitude faster than the rate at which veratridine unbinds at −90 mV; (*c*) the extent to which modified channels shut depends on voltage, with smaller hyperpolarizations resulting in less shutting.

The behavior of veratridine-modified channels resembles normal activation gating in that modified channels can be made to open on depolarization and shut on hyperpolarization. However, it differs from normal activation gating in three ways: (*a*) the voltage range over which gating of modified channels occurs is much more negative than the voltage range over which normal gating occurs; (*b*) the opening and shutting of modified channels is much slower than that of unmodified channels; (*c*) the opening of modified channels does not have the sigmoidal time course shown by normal Na channels. We can call the shut state "VS" and add it to the proposed model, which becomes:



Inactivation of Veratridine-modified Channels

As shown in Fig. 3, during a series of depolarizing pulses, the veratridine-induced tail current increases, reaches a maximum, and may subsequently undergo a slow decrease. The decrease in inward-flowing current could be due to an accumulation of Na ions in the muscle fiber caused by the large, continuous inward current, or by a slow inactivation of the channels during the repetitive pulsing. One way to choose between these possibilities is to increase the length of the depolarizing pulses while keeping the rate of pulsing the same. This decreases

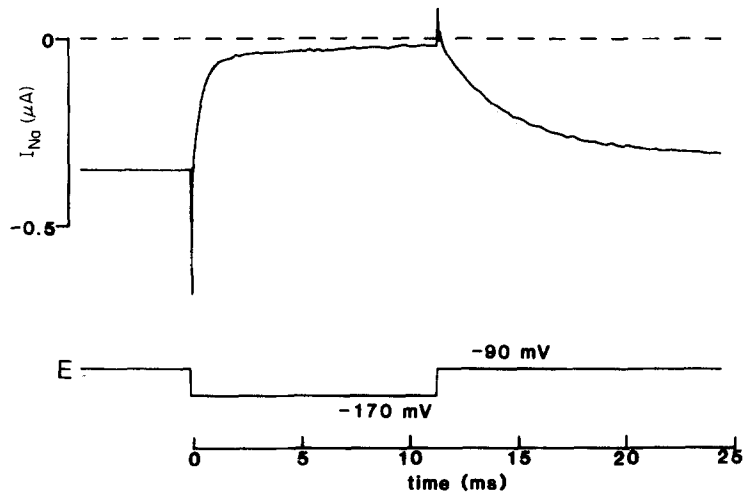
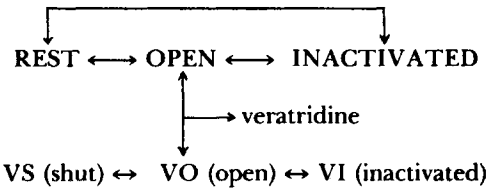


FIGURE 9. Hyperpolarization shuts veratridine-modified channels. This fiber was equilibrated in 100 μM veratridine Ringer at a circulating coolant temperature of 8°C and a holding potential of −90 mV. The output current was filtered at 10 kHz. A series of 20 depolarizations to +6 mV was applied to the fiber to obtain a large tail current. Then the fiber was held at −90 mV for 5 ms, followed by a 5-ms depolarization to +6 mV, and a return to −90 mV for 50 ms. The average of the final 50 ms of this period is shown at the beginning of the trace in the figure followed by an 11.25-ms hyperpolarization to −170 mV, and finally 13 ms at −90 mV. In this experiment, separate electrodes were used for measuring voltage and passing current, the command pulses were computer-generated, and the currents were recorded on computer tape.

the time the fiber spends at −90 mV, where the tail current is large, and increases the time at the depolarized potential, where a voltage-dependent slow inactivation process might be developing. In short, with an appropriate choice of potential and duration, the Na accumulation in the fiber may be lessened, and possible inactivation may be enhanced.

An experiment of this type is shown in Fig. 10. Increasing the length of the depolarizing pulses from 12 to 72 ms enhances the slow decrease in veratridine-induced current, the opposite of what the accumulation hypothesis predicts. Therefore, it is probable that a slow inactivation occurs in modified Na channels. This process is more rapid and complete than the slow inactivation that occurs

when identical experiments are performed on untreated fibers. The inactivation seems to depend on voltage since it becomes more pronounced at higher voltages or with longer depolarizing pulses. Adding this inactivated state to the hypothesis gives the kinetic scheme:



Since I have never seen the Na current reach zero during repeated pulsing, I cannot say whether the Na channel is nonconducting in the VI state or retains some residual conductance.

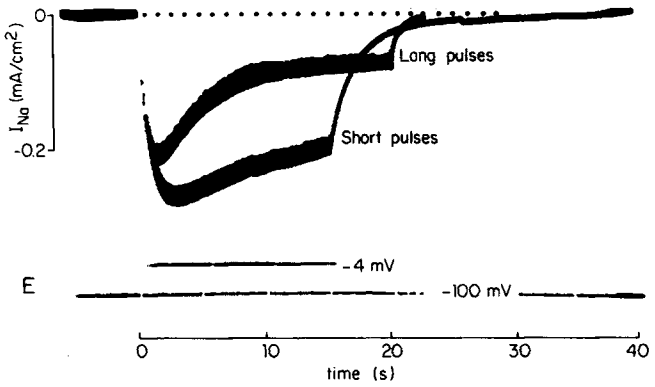


FIGURE 10. Longer test pulses result in more pronounced slow inactivation. The fiber was equilibrated in 50 μM veratridine Ringer and cooled with an 11°C circulating coolant. Test pulses with a duration of either 12 or 72 ms were applied at a frequency of 9 Hz. The time scale is slow so that either 135 or 180 individual pulses are included on each trace, and the individual voltage pulses appear to blend into solid lines at a pulse potential of −4 mV and a holding potential of −100 mV. The current flowing during the pulses is too fast to be seen, but what can be seen are the tail currents. The solution in the B, C, and E pools was 120 mM CsF.

Modification of Channels Represents Veratridine Binding and Unbinding

Thus far, I have considered the development of a pharmacological effect as a direct measure of the binding or unbinding of veratridine. It is conceivable, however, that veratridine remains bound to the Na channel throughout the experiment, but is originally in an "inactive," bound position, until the channel opens and allows the drug to shift into a new configuration, where it holds the channel open. In such a model, the veratridine concentration in the bathing solution determines only how many channels have veratridine already bound. The probability of going into the modified configuration is the same for all channels with bound veratridine. Therefore, although the size of the veratridine-

induced current would depend on the veratridine concentration, the normalized time course of the development and the inactivation of veratridine-induced current would not.

This alternative is tested in Fig. 11, where identical pulse sequences are applied to fibers bathed in either 1.2 or 98 μM veratridine Ringer. An increase in the veratridine concentration does increase the maximum size of the veratridine-induced current, but it also shortens the time to peak and makes the subsequent decline during the pulse train pronounced. The rate at which the veratridine-

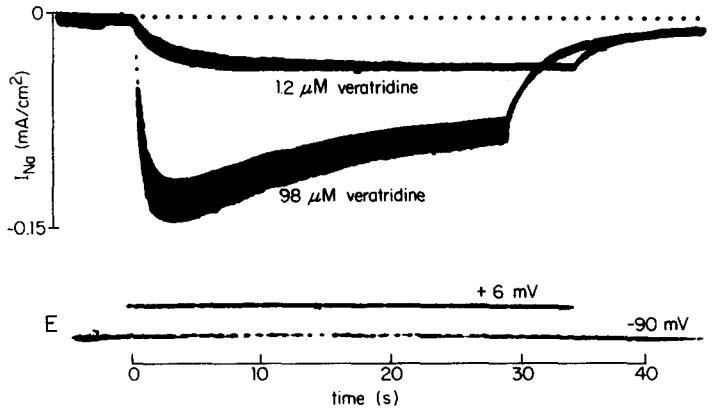


FIGURE 11. Apparent dependence of slow inactivation on veratridine concentration. The fiber was first equilibrated in 1.2 μM veratridine Ringer and a series of 9-ms depolarizations from −90 to +6 mV was applied at a pulse rate of 12.5 Hz. The fiber was subsequently equilibrated in 98 μM veratridine Ringer, and subjected to the same pulse paradigm. The time scale used is slow, so that the voltage pulses blend together and appear to be solid lines at +6 and −90 mV. The current during pulses is too fast to be seen, and the figure shows only the tail current between pulses and after cessation of pulsing. The B, C, and E pools contained 120 mM CsF.

induced current returns to zero after the cessation of pulsing seems to be unaffected. My qualitative results require that additional veratridine binding occur during the experiment, although they could still be consistent with a two-step model where binding is slow enough not to occur during a pulse but fast enough to occur during the interpulse interval. The following paper (Leibowitz et al., 1986) gives arguments against the two-step model.

DISCUSSION

Comparison with Earlier Work

I conclude that: (*a*) Veratridine binds readily to open Na channels, but poorly, if at all, to channels in the resting or inactivated states. (*b*) Bound veratridine causes channels to be held open, so that they neither undergo normal fast inactivation nor shut upon return to the normal resting potential. (*c*) At a membrane potential of −90 mV, veratridine unbinds from Na channels, with a time constant of ~3 s. The cycle of binding and unbinding can be repeated many

times. (*d*) Chemical agents that slow inactivation enhance the binding of veratridine by allowing channels to remain open longer than usual. (*e*) Veratridine-modified channels shut when the membrane is hyperpolarized from the normal resting potential and reopen at the normal resting potential. This process may be a modified form of normal activation gating. (*f*) Veratridine-modified channels are subject to a very slow, voltage-dependent inactivation process.

Several of the effects described here have not previously been reported for veratridine but have been seen using other alkaloid neurotoxins. Series of depolarizing pulses or action potentials have been reported to enhance the effects of BTX (Khodorov, 1978; Khodorov and Revenko, 1979), aconitine (Herzog et al., 1964; Mozhayeva et al., 1977; Campbell, 1982), grayanotoxin (Seyama and Narahashi, 1981), and veratridine (Ulbricht and Flacke, 1965; Meves, 1966; Ulbricht, 1969; Leicht et al., 1971*a*). These studies indicate that the binding of BTX, aconitine, and grayanotoxin to Na channels is nearly irreversible. Ulbricht (1972*b*) reports that veratridine can be completely washed out only after short applications.

BTX holds Na channels open at the normal resting potential and removes Na channel inactivation. Modified channels show a voltage-dependent activation that is slower than normal activation, has single-exponential kinetics, and appears at potentials hyperpolarized by ~40–50 mV with respect to normal Na channel activation (Khodorov et al., 1975; Khodorov, 1978). Similar effects have been reported for grayanotoxin (Seyama and Narahashi, 1981) and for aconitine (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976, 1981; Campbell, 1982).

Veratridine is grouped with the other alkaloid neurotoxins on the basis of pharmacological experiments on the uptake of radioactive Na ions by vesicles containing Na channels (Catterall, 1975*a*, *b*, 1977*b*), but voltage-clamp studies (Ulbricht, 1969; Leicht et al., 1971*a*, *b*) have suggested only remote similarities between the actions of veratridine and those of the other alkaloid neurotoxins. These studies using long depolarizing pulses found an inward Na current that developed over several seconds with a voltage dependence that was similar to that of normal activation and that either did not inactivate (Ulbricht, 1969) or inactivated slowly and incompletely (Leicht et al., 1971*b*).

The development and disappearance of this current was interpreted by Ulbricht and by Leicht et al. as the slow opening and shutting of veratridine-modified channels. I would interpret it as reflecting the slow binding and unbinding of veratridine from the Na channel. During a long depolarization, there is a spurt of veratridine binding while Na channels are in the open state, followed by a much slower binding during the rest of the pulse. This slow binding could be due either to a slow binding of veratridine to inactivated Na channels, or to a small fraction of inactivated channels returning to the open state, where they are available for veratridine binding. My experiments would not distinguish between the two possibilities. They are not mutually exclusive, and the observed effect could easily be due to some combination of the two. The second possibility would explain the observed slow rates of binding (Ulbricht, 1969; Leicht et al., 1972*b*; Ducreux and Gola, 1982) if, on the average, only $\leq 2\%$ of the channels were open during a long depolarization.

Ulbricht (1965, 1969), Hille (1967), and Leicht et al. (1971*a*) noted persistent

Na currents after single, short voltage pulses, and Ulbricht observed that the tail currents increase with repeated pulsing. In analogy with his work on DDT, Hille (1968) first suggested that these tail currents indicate that veratridine binds only to open channels. A similar model has been proposed independently by Gola et al. (1982) and Ducreux and Gola (1982), in which open Na channels rapidly become veratridine-modified, while shut Na channels do not become modified, and inactivated Na channels become modified only very slowly. In their model, modified channels do not shut or undergo normal fast inactivation, but do undergo a very slow (τ = 21.5 s) inactivation.

Krueger and Blaustein (1980) and McKinney (1982) have proposed that veratridine binds predominantly to the open state and less avidly to the inactivated state. Scruggs and Landowne (1978) conclude from experiments on internally perfused squid giant axon and results similar to mine that "veratridine interacts with the normal Na channel, modifies its kinetics, and possibly its selectivity." Scruggs and Narahashi (1982) suggest that "both closed and open states are precursors to the veratridine-modified open state," while Yoshii and Narahashi (1984) suggest that veratridine binds predominantly to the open state.

There is an apparent contradiction between my observation that veratridine appears to bind more rapidly at more depolarized membrane potentials and the conclusion by Ulbricht (1972*b*) that "the rate of veratrinization appeared to be independent of membrane potential." Ulbricht's conclusion comes from experiments in which he applied veratridine to frog node of Ranvier hyperpolarized by 12 mV or depolarized by 33 mV from the normal resting potential and found that a test pulse induced the same slow veratridine-induced current in both cases (Ulbricht, 1972*b*, Fig. 5). I believe that there is not really a contradiction, since my experiments show that veratridine binds afresh to Na channels when they open and unbinds at the normal resting potential. Therefore, as far as veratridine binding is concerned, it does not matter what the membrane voltage is when the drug is first applied. What does matter is the pulse paradigm used to elicit and observe the veratridine-induced currents. Since Ulbricht used the same pulse paradigm to elicit control currents and experimental currents, his experiments did not really test whether veratridine binding is voltage dependent.

My work and the studies cited above show that the effects of veratridine parallel those of the other alkaloid neurotoxins: veratridine binds preferentially to open Na channels and, when bound, removes fast inactivation. The voltage dependence of activation gating is shifted to more negative potentials, and the gating kinetics are altered. The major difference from the other alkaloid neurotoxins is that veratridine unbinds from Na channels in seconds, while the others stay bound for hours. The apparent slow inactivation, seen in veratridine-modified Na channels by me and others (Leicht et al., 1971*b*; Ducreux and Gola, 1982), has not been reported in Na channels modified by BTX or aconitine, although a fast inactivation has been reported for aconitine (Mozhayeva et al., 1981; Campbell, 1982). Seyama and Narahashi (1981) have found evidence for a small, slow inactivation in grayanotoxin-treated Na channels, and long pulse experiments might reveal a slow inactivation in Na channels modified by BTX or aconitine (but see Khodorov et al., 1975).

Several other substances have effects similar to those of the alkaloid neurotoxins. DDT binds only to open channels, slows inactivation in such channels, and holds the channels open upon return to the resting potential (Hille, 1968; Århem and Frankenhaeuser, 1974; Dubois and Bergman, 1977; Lund and Narahashi, 1981*a*; Leibowitz et al., 1986). Similar results have been obtained using a number of synthetic compounds that are only distantly related to DDT, such as 2,4,6-trinitrophenol (Oxford and Pooler, 1975) and the pyrethroid insecticides (Lund and Narahashi, 1981*b*; Vijverberg et al., 1982, 1983; Leibowitz et al., 1986).

Another toxin with similar actions is the venom from the scorpion *Centruroides sculpturatus*, which modifies Na channels during a depolaizing pulse, so that they do not shut at the normal resting potential, but retain nearly normal inactivation characteristics (Cahalan, 1975). Modified channels exhibit an activation gating that is shifted to the negative by ~40–50 mV with respect to the activation of normal channels. Wang and Strichartz (1982) found that *Leiurus* scorpion toxin enhances *Centruroides* toxin–induced tail currents in a manner reminiscent of the enhancement of veratridine-induced tail currents by *Leiurus* toxin. These effects of *Centruroides* venom bear a strong resemblance to the effects of veratridine, as noted by Hille (1984).

Removal of Inactivation Enhances Veratridine Binding

The ability of scorpion toxin to potentiate the action of veratridine has been observed by several investigators (Catterall, 1975*b*, 1977*b*; Stallcup, 1977; Romey et al., 1979; Jacques et al., 1978; Tamkun and Catterall, 1981), using various combinations of Na flux assays, scorpion toxin binding assays, and voltage-dependent fluorescent dyes. Enhancement of scorpion toxin binding by veratridine and BTX has also been demonstrated (Catterall, 1977*a*; Tamkun and Catterall, 1981), although I have not been able to investigate this phenomenon electrophysiologically.

My experiments indicate that *Leiurus* toxin enhances veratridine binding by holding the channels open longer, thereby increasing the availability of the channels for veratridine binding. All the inactivation-removing agents I tested (NBA, *Leiurus* toxin, chloramine-T, low internal pH) had similar effects, although they had few other similarities. Additionally, Scruggs and Narahashi (1982) found that internal pronase enhances veratridine effects in squid giant axon. Wang and Strichartz (1983) concluded that chloramine-T "does not potentiate veratridine's ability to depolarize the resting membrane," which is in apparent contradiction to my observations. I believe, however, that this apparent contradiction is due to the different measures of veratridine effects that we used.

It is likely that any agent that removes or slows inactivation, except another alkaloid neurotoxin, will enhance veratridine binding. I propose that the ability of *Leiurus* toxin and other inactivation-removing agents to potentiate the effects of BTX, aconitine, and grayanotoxin is also due to the *Leiurus* toxin holding the channel in the open state for a longer time, allowing more opportunity for the alkaloid to bind. Similar effects occur with *Centruroides* toxin (Wang and Strichartz, 1982), DDT, and allethrin (Leibowitz et al., 1986) and would be expected for the other DDT analogues.

Does Veratridine Binding Precede Na Channel Opening?

As noted above (see Figs. 4 and 7), when the normalized time integral of Na current during a depolarizing pulse is compared with the normalized tail currents after pulses of various lengths, the tail currents apparently develop more rapidly than the integral early in the pulse. The deviation is reduced, but not eliminated, by correcting at each time for the Na channels that already have veratridine bound to them. One possibility is that veratridine binding actually does precede the opening of Na channels, which indicates that veratridine has some ability to bind to Na channels in one or more of the states intermediate between the rest and open states, as well as to Na channels that are open. Another possible explanation is that veratridine binds to unmodified Na channels before they have time to shut after the return to −90 mV. The effect of this binding will be proportionately larger for short depolarizations when binding during the pulse is small.

In order to estimate this error, I calculated the time integral of the shutting of unmodified Na channels during the tail by multiplying the time constant for the shutting of unmodified channels by the total current through the unmodified Na channels at the beginning of the tail. These integrals were added to the Na current integral. Even after this correction, the tail currents still preceded the Na current integral slightly, although the effect was reduced compared with before. This was also true of fibers treated with veratridine plus either NBA or *Leiurus* toxin. The evidence suggests that the binding of veratridine can slightly precede the opening of Na channels, but at the present time the evidence is inconclusive.

Possible Sources of the Apparent Voltage Dependence of Veratridine Binding

Binding is apparently voltage dependent (Fig. 10). According to the hypothesis proposed here, veratridine binds only to open channels, so if binding were voltage independent, veratridine-induced tail currents would vary with voltage in the same manner as the integral of Na permeability. This is not what was observed. Veratridine-induced tail currents increased monotonically with increasing voltage, while the integral of Na permeability first increased with increasing voltage, but then dropped off again. One possible explanation is that veratridine might bind to inactivated channels during large depolarizations. As can be seen in Fig. 6, there appears to be some continued binding after inactivation is complete. The maximum observed increase in veratridine-induced tail currents after inactivation is complete is ~17%, however, and even adding in 5% for data scatter, there is still an increase of only 22%, whereas an increase on the order of 100% would be required to explain the observed discrepancy.

Since the veratridine molecule is uncharged, it is difficult to conceive of how the voltage dependence could be a property of the veratridine molecule itself. The voltage dependence could be due to a voltage dependence of the veratridine-binding site. This can be modeled by assuming that the rate of binding to open channels increases exponentially with increasing voltage. Alternatively, the apparent voltage dependence of binding might come from the gating properties of the Na channel. One way this can be modeled is to assume that the channel has

a probability of opening from any one of a number of "permissive" states. The transitions between these permissive states are voltage dependent, and both the probability of channel opening and the probability of veratridine binding depend on which of these permissive states the channel is in. A model of this sort, based on that of Hoyt (1984), was able to reproduce most of the qualitative features of my data (Sutro, 1984*a*).

The major thrust of this paper has been to establish that veratridine binds to the open state of the Na channel and that veratridine-modified channels can enter shut and inactivated states. The following paper (Leibowitz et al., 1986) examines the transitions between these states of the veratridine-modified Na channels, and compares veratridine with other drugs having similar effects.

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