

Voltage-dependent Gating of Veratridine-modified Na Channels

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ABSTRACT Na channels of frog muscle fibers treated with 100 μ M veratridine became transiently modified after a train of repetitive depolarizations. They open and close reversibly with a gating process whose midpoint lies 93 mV more negative than the midpoint of normal activation gating and whose time course shows no appreciable delay in the opening or closing kinetics but still requires more than two kinetic states. Like normal activation, the voltage dependence of the modified gating can be shifted by changing the bathing Ca^{2+} concentration. The instantaneous current-voltage relation of veratridine-modified channels is curved at potentials negative to -90 mV, as if external Ca ions produced a voltage-dependent block but also permeated. Modified channels probably carry less current than normal ones. When the concentration of veratridine is varied between 5 and 100 μ M, the initial rate of modification during a pulse train is directly proportional to the concentration, while the rate of recovery from modification after the train is unaffected. These are the properties expected if drug binding and modification of channels can be equated. Hyperpolarizations that close modified channels slow unbinding. Al-lethrin and DDT also modify channels. They bind and unbind far faster than veratridine does, and their binding requires open channels.

INTRODUCTION

This work was undertaken to pursue the parallels between the well-described electrophysiological actions of batrachotoxin (BTX) and aconitine and the less well-described actions of veratridine and other lipid-soluble toxins. The preceding paper (Sutro, 1986) shows that veratridine reacts with Na channels only when they are open, producing a modified state of the channel that still opens and shuts in response to voltage steps. Here we ask whether this modified gating is similar to the shifted activation gating that has been reported for aconitine-, BTX-, and grayanotoxin-treated Na channels (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976, 1981; Khodorov et al., 1975; Khodorov and Revenko, 1979; Seyama and Narahashi, 1981; Campbell, 1982). We probe further the hypothesis that veratridine binding and channel modification should be equated

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and ask whether the insecticides bis(chlorophenyl)trichloroethane (DDT) and allethrin also react with Na channels only when the channel is open. A preliminary report has appeared (Leibowitz et al., 1985).

METHODS

Preparation and Recording

Sections of single skeletal muscle fibers from *Rana pipiens* semitendinosus muscles were isolated and mounted for study under voltage-clamp conditions as described previously (Sutro, 1986; Dani et al., 1983; Hille and Campbell, 1976). The voltage clamp, designed by Dr. W. Nonner (University of Miami, Miami, FL), had separate current and voltage electrodes in the recording pool to reduce the effects of series resistance. Current records were corrected for linear leakage and capacitive currents, using a manually adjusted analog transient generator driven by the command potential, before filtering with an active four-pole, low-pass Bessel filter. The current signal was then sampled digitally and stored on magnetic tape for later analysis. All stimulus and digital sampling pulses were generated by a locally built digital stimulator, which was programmed by our LM² minicomputer (Kehl et al., 1975), and the minicomputer was used to digitize, store, and analyze the data. The membrane current and voltage were also monitored on a stripchart recorder. Analysis programs included a nonlinear least-squares fitting routine based on the Gauss method. In some figures plotting mean values, error bars are shown representing ± 2 SEM if the error is larger than the size of the symbol.

The plastic chamber containing a muscle fiber fragment was allowed to equilibrate in the recording chamber at 9°C for 10–20 min before the cooled agar bridges were inserted, the test (A) pool solution was changed to Ringer (115 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, and 4 mM morpholinopropanesulfonic acid [MOPS], pH 7.2), and recording was begun. All other pools contained 115 mM CsF and 5 mM NaCl to eliminate K currents and muscle contractions and to uncouple the transverse tubular membrane system from the surface membrane (Campbell and Hahn, 1983; Campbell, 1984). We agree with the Campbell laboratory that the slow component of membrane capacity current ($\tau = 1\text{--}4$ ms) is gradually lost in fluoride-treated fibers once Ringer is introduced into the test pool.

Veratridine (Aldrich Chemical Co., Milwaukee, WI) was dissolved in acidified Ringer, which was then returned to normal pH (7.2) using NaOH. Allethrin (allethrin I, kindly provided by Dr. Toshio Narahashi, Northwestern University, Chicago, IL) and DDT (Nutritional Biochemicals Corp., Cleveland, OH) were dissolved as 20- or 25-mM solutions in ethanol and diluted into Ringer immediately before use. Except where otherwise noted, the final concentrations used were: 100 μM veratridine, 200 μM allethrin, and 250 μM DDT. Upon addition to Ringer, the DDT precipitated to form a milky suspension; therefore, although the DDT concentration was calculated to be 250 μM , in actuality the applied concentration was unknown but saturated. The final concentration of ethanol was never greater than 1%. *N*-Bromoacetamide (NBA; Sigma Chemical Co., St. Louis, MO) was dissolved in Ringer the day it was to be used.

Induction of the Drug-modified State

As the previous paper describes (Sutro, 1986), repetitive stimulation in the presence of veratridine induces a modified state of Na channels that persists for several seconds at a -90-mV holding potential. In most experiments reported here, we generated a population of modified channels by conditioning pulse trains and then studied the channels in the following 150 ms before many had reverted to the normal state. Conditioning usually

involved 10 10-ms pulses to +6 mV applied at 4 Hz. The holding potential was always -90 mV. As we show later, DDT and allethrin produced modified states that reverted so rapidly to the normal state that no benefit was obtained by applying more than one conditioning pulse.

RESULTS

Voltage-dependent Gating of Veratridine-modified Channels

Veratridine-modified Na channels shut rapidly when the membrane is strongly hyperpolarized and reopen when the potential is returned to -90 mV (Sutro, 1986). We have examined the kinetics of the closing and reopening processes at potentials ranging from -170 to -74 mV in steps of 8 mV. Fig. 1 shows observations with a fiber exposed to 100 μ M veratridine. A train of conditioning depolarizations was applied immediately before the recordings were begun in order to produce a population of modified channels. Their presence is reflected in the appreciable inward Na current seen at the beginning of each frame, where the membrane potential is at -90 mV. Each subsequent sweep was preceded by a 10-ms depolarization to +6 mV to maintain the population of modified channels. Fig. 1A is a "closing experiment" with steps to various hyperpolarizing potentials. During a hyperpolarization to -122 mV, the inward current declined by ~50% in 15 ms, and during a hyperpolarization to -170 mV, the inward current fell almost to zero in 1 ms. Fig. 1B is a "reopening experiment" with steps to various potentials following a -170 mV hyperpolarization that closed most of the channels. Depolarization from -170 to -90 mV reopened most of the channels in 15 ms, and depolarization to -114 mV reopened about half the channels.

Steady state. These experiments have been analyzed in two ways, for steady state values and for time course. We begin with the steady state gating parameters extracted from the closing experiments. Analysis of the raw current records raised several problems. First, the deduced degree of closing at very negative potentials is sensitive to appropriate leak subtraction. Therefore, once we had recorded currents in modified channels, the same fiber was bathed with a solution containing 100 μ M veratridine and 156 nM tetrodotoxin (TTX) to block Na channels, and the pulse protocol was repeated. The kinetic analysis was then done on difference currents. Another problem was that each trace began with a slightly different number of modified Na channels. To eliminate this $\pm 5\%$ variability, we normalized individual traces with respect to the starting current at -90 mV. The traces drawn in Fig. 1 have already been scaled this way. The final problem was that we desired the steady state fraction of open channels at each voltage rather than the steady state current. To avoid making assumptions about the current-voltage relations of single channels (described later), we chose to measure the fraction of channels from the size of the tail currents upon the return to -90 mV. Such tails are shown starting at the 47-ms time point in Fig. 1A. They were well resolved and monotonically increasing, so that extrapolation to zero time using a straight line fitted to the first 150 μ s or an exponential plus a constant fitted to the first 700 μ s gave identical values.

The fraction of open, modified Na channels after a 47-ms "closing pulse" to various potentials is shown in Fig. 2A (open circles). The smooth curve is a least-squares fit of a Boltzmann function,

$$F = \{1 + \exp[(E - E_{0.5})/k]\}^{-1}, \quad (1)$$

with a midpoint $E_{0.5} = -114$ mV and a slope factor $k = 10.6$ mV. As others have suggested from experiments with BTX and aconitine, we view the gating of

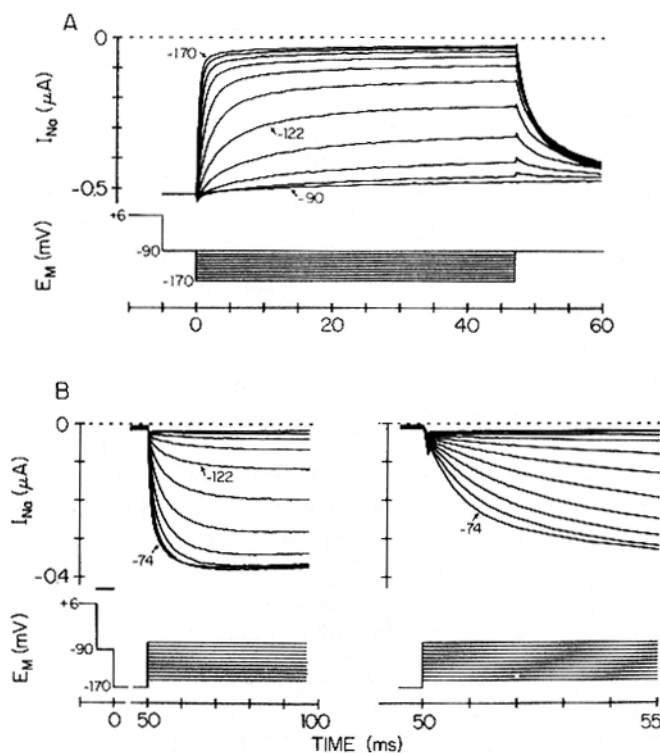


FIGURE 1. Voltage-dependent closing and reopening of veratridine-modified channels. A single muscle fiber was bathed in Ringer containing $100 \mu\text{M}$ veratridine and held at a holding potential of -90 mV. Each series began with a conditioning train of 10 depolarizing pulses (not shown) to produce a population of modified channels. Then hyperpolarizing pulses were added to study the gating of these modified channels. The superimposed traces have been slightly normalized to make the initial currents at -90 mV identical. A current of $0.5 \mu\text{A}$ corresponds approximately to a current density of $1 \text{ mA}/\text{cm}^2$. (A) After the conditioning train, the following sequence of steps was applied every 275 ms: $+6$ mV for 10 ms, -90 mV for 5 ms, from -90 to -170 mV in intervals of 8 mV for 47 ms (to observe closing kinetics), and back to -90 mV. (B) After a conditioning train, the following sequence was applied every 312 ms: $+6$ mV for 10 ms, -90 mV for 5 ms, -170 mV for 50 ms, from -154 to -74 mV in intervals of 8 mV for 47 ms (to observe reopening), and back to -90 mV. B shows the same records drawn at two different sweep speeds. The analog signals were filtered at 10 kHz.

veratridine-modified channels as a modified form of the activation gating steps, occurring, however, at more negative membrane potentials and with slower kinetics than in normal fibers. For comparison, the diamonds in Fig. 2A show the peak fraction of open Na channels before veratridine treatment. The curve drawn is the same as that for modified channels, except that the midpoint is shifted. Closer analysis of these observations indicates three differences between the steady state activation of modified channels and peak activation of normal channels. For modified channels, the midpoint of activation is shifted by -93 mV, the Boltzmann curve provides a better overall fit, and the steepness of the voltage dependence is less. These quantitative conclusions are reached whether the analysis is done on averaged activation curves or fiber by fiber. A similarity

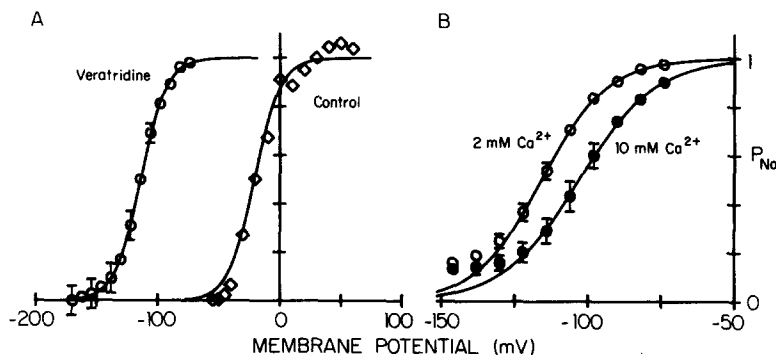


FIGURE 2. Voltage dependence of activation gating. (A) Comparison of veratridine-modified and normal channels. The diamonds show the peak Na permeability at each potential in a normal muscle fiber. The circles, for modified channels, are means ($N = 6$) of extrapolated instantaneous current amplitudes at -90 mV after 47-ms closing pulses (protocol of Fig. 1A but after subtraction of currents insensitive to TTX). Error bars (± 2 SEM) are shown only on every other point. The smooth curves are from Eq. 1 with $k = 10.6$ mV and $E_{0.5} = -114$ and -21.2 mV. (B) Comparison of modified channels in normal (2 mM, open circles) and high (10 mM, filled circles) bathing Ca^{2+} measured in the same fibers ($N = 6$). The curves are drawn with $k = 11.2$ mV and $E_{0.5} = -116$ mV for normal $[\text{Ca}^{2+}]$ and 13.0 mV and -103 mV for high $[\text{Ca}^{2+}]$.

between modified and normal activation gating is that both can be shifted by changing the concentration of Ca ions in the bath. Thus, when the external Ca^{2+} concentration is increased fivefold from 2 to 10 mM, $E_{0.5}$ for normal channel gating would be expected to be shifted $+13.2$ mV (Campbell and Hille, 1976). For veratridine-modified channels, it is shifted $+13$ mV (Fig. 2B). The points in Fig. 2B were calculated from records that had not been corrected with the TTX method, and the apparent lack of full closing at negative potentials may be artifactual.

In other experiments not shown, fibers were treated with 1 mM NBA to slow the inactivation of Na channels, as in the previous paper (Sutro, 1986). In frog muscle, NBA has very little effect on the voltage dependence of normal activation

gating (Nonner et al., 1980). Similarly, in our experiments, it also had little effect on the steady state activation curve of veratridine-modified channels.

Time course. We turn now to the time course of modified activation gating recorded as in Fig. 1. At every potential, the closing and reopening kinetics have fast and slow components. The curves were well fit by two exponential components and only poorly by one. Fig. 3 shows fast and slow components resolved by curve fitting to the time course of closing at -170 mV and the time course of reopening at -74 mV. For either trace, the amplitudes of the two exponentials had the same sign and significant magnitude. Except for small repolarizations from -170 mV, where the currents were small and poorly resolved, the closings

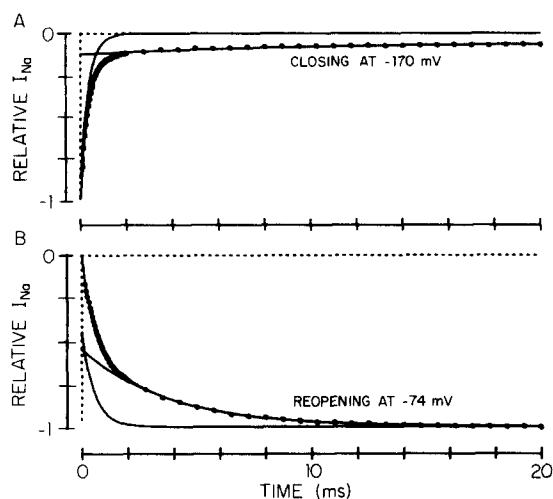


FIGURE 3. Resolution of closing and reopening time courses into two exponential components. The measurements are shown as filled circles. The three solid lines are the least-squares-derived fast and slow exponential components and their sum. The dashed lines indicate zero current and zero time. Same experiment as Fig. 1. (A) Closing time course at -170 mV. The time constants and relative amplitudes of the fitted components are 0.35 (93%) and 9.6 (7%) ms. (B) Reopening at -74 mV after a closing at -170 mV. The time constants and relative amplitudes are 0.53 (54%) and 3.7 (46%) ms.

or reopenings clearly began without prominent delays or hooks and proceeded at the maximum rate within $150 \mu\text{s}$ of the beginning of the voltage step (Fig. 1C). In this respect, the reopening of modified channels differs from the sigmoid activation kinetics typical of normal Na channels. The mean values of the fitted time constants from such fits are summarized in Fig. 4. The fast time constants for opening and closing seem to form a single curve, peaking near -120 mV, increasing e-fold in 57 mV on the negative side, and decreasing e-fold in 110 mV on the positive side. The voltage dependence of the slow opening and closing time constants appears weaker and not bell-shaped; they seem not to describe a single curve, as if they represented more than one poorly resolved, slow process. As we observed in the steady state measurements, when the muscle fibers were

treated with NBA to slow inactivation (not shown), the time constants were qualitatively unchanged. In addition, when the bathing Ca^{2+} concentration was raised fivefold, all time constants were shifted by approximately +12 mV along the voltage axis.

The multiexponential nature of gating in modified channels is again evident in Fig. 5. Here the membrane potential was stepped from -90 to -120 mV, and gradually about half the modified channels closed. This pulse was interrupted at various times by steps to -170 or -90 mV, where channels close or reopen fully with clear double-exponential kinetics. Notice, however, that the time course of closing or reopening was changed by the step to -120 mV. The fraction of

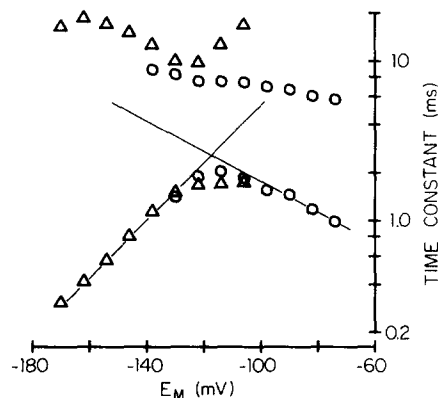


FIGURE 4. Voltage dependence of opening and closing time constants for veratridine-modified channels. Current traces generated by the two protocols of Fig. 1 were fitted by the sum of two exponentials as in Fig. 3. The slow and fast reopening time constants (circles) and the slow and fast closing time constants (triangles) are plotted semilogarithmically. They are the means of five to eight observations each. The lines represent an e -fold increase in 57 mV and an e -fold decrease in 110 mV. All symbols are larger than ± 1 SEM, except for the two largest values of the fast opening time constant.

channels closing rapidly at -170 mV or reopening rapidly at -90 mV decreased as the step to -120 mV was lengthened. Another surprising observation was that after a pulse to -120 mV as brief as 2 ms, a small but significant fraction of channels failed to reopen at -90 mV (Fig. 5). We have been able to imitate all of these kinetic features with models having two modified open states, VO, and one or two modified shut states, VS, connected in a topology $\text{VO}_2\text{-VO}_1\text{-VS}$ or $\text{VS}_2\text{-VO}_2\text{-VO}_1\text{-VS}_1$, where VO_1 is favored at -90 mV and at more depolarized potentials, VS_2 and VO_2 are favored at -120 , and VS_1 is favored at -170 mV. However, without further information, it would be difficult to rule out other possibilities.

Permeability Properties of Veratridine-modified Channels

The modified channels offer a chance to study permeation in a voltage range where Na channels are normally shut. The instantaneous current-voltage curve

of veratridine-modified Na channels is clearly nonlinear. For example, in Fig. 1A, at the moment when the membrane potential was stepped from -90 to -170 mV, I_{Na} hardly changed, although the driving force on Na ions was increased by perhaps 60%. Therefore, we designed experiments to measure the instantaneous current-voltage relations of modified channels over a broad voltage range (Fig. 6). After a conditioning train, the current was measured at -40 mV, where unmodified channels remain inactivated, and then at a second potential extrapolated back to the time of the step (see sample traces). The instantaneous current-voltage relation was N-shaped at very negative potentials (open circles). Much of this curvature seems to come from an instantaneous, voltage-dependent

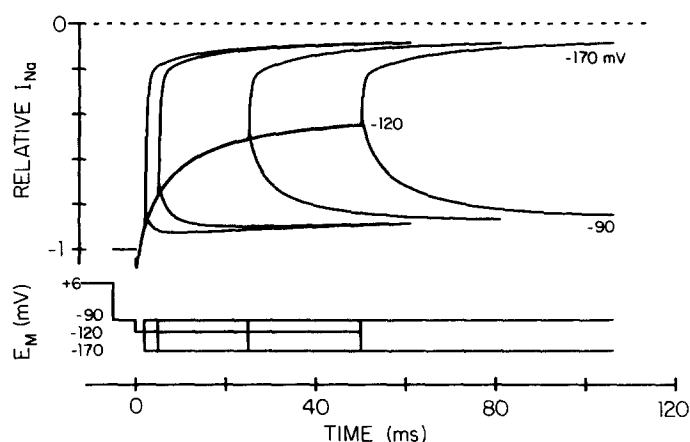


FIGURE 5. Time courses of closing and reopening after partial closing of veratridine-modified channels. After a series of conditioning pulses to induce a population of modified channels, the following pulse sequences were applied: $+6$ mV for 10 ms, -90 mV for 5 ms, -120 mV for 2, 5, 25, and 50 ms, -170 or -90 mV for 56 ms, and back to -90 mV for 200 ms. All traces have been normalized to the initial current at -90 mV, which corresponds to a current density of approximately -1.4 mA/cm².

block of Na channels by external Ca ions, because when the bathing Ca²⁺ concentration was raised fivefold, currents were severely reduced between -90 and -170 mV (closed circles). We did not perform experiments in low-Ca²⁺ solutions because the muscle membrane does not survive well such intensive trains of pulses without Ca ions. The block seems to be relieved by the strongest hyperpolarizations, as if Ca ions were finally forced through the channel by the strong applied electric field. In 2 mM Ca²⁺, the tail currents at potentials more negative than -145 mV decay so rapidly that extrapolation to zero time becomes a questionable procedure, but in the high-Ca²⁺ solution, the extrapolation is clearer and indicates relief of block by hyperpolarization.

We have not explicitly looked for ionic selectivity changes in modified channels. Although the apparent reversal potential ($+40$ mV) in Fig. 6 is lower than values of $+70$ mV typical of these experiments before veratridine treatment, we are not confident that this indicates a decrease of ionic selectivity. Repetitive stimu-

lation and standing inward currents in modified channels lead to increased Na loading of the fiber and hence a lowered reversal potential. Also, the modified channels do not have the transient open-close kinetics that help to confirm in experiments with normal channels that leak and capacity subtraction have been done correctly. It would be preferable to make selectivity measurements at very negative potentials, where contributions from normal channels would be absent.

We also wished to determine whether modified channels have a different single channel current from normal channels. This cannot be done with certainty without using microscopic techniques, but we can nonetheless make relevant

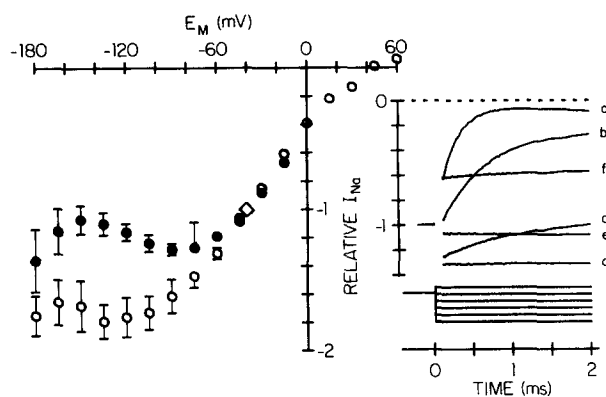


FIGURE 6. Instantaneous current-voltage relations of veratridine-modified Na channels. Modified channels were generated by a conditioning train of depolarizing pulses and then the following sequence of steps was applied every 222 ms: +6 mV for 10 ms, -40 mV for 10 ms (normalization), -180 to +60 mV in increments of 15 mV for 2 ms (test), and back to -90 mV. (Left) Current-voltage relation with the current at -40 mV in each sweep normalized to 1.0 (diamond) and the relative instantaneous current at each test step extrapolated back to the time of the transition. Currents were measured in 2 mM (open circles, $N = 11$) and 10 mM (filled circles, $N = 6$) external Ca^{2+} concentrations. (Right) A sample of records in 2 mM Ca^{2+} showing the end of the pulse to -40 mV followed by test steps to -165, -135, -105, -75, -45, and -15 mV, labeled *a-f*, respectively. Data points at 25, 50, 75, and 100 μs after the transitions have been deleted. Filter frequency, 12.6 kHz.

macroscopic measurements. Consider currents measured in successive pulses of a conditioning train (Fig. 7), focusing on values (*a*) at the peak, (*b*) late in the pulse, and (*c*) in the tail. As more channels become modified, the peak current becomes smaller (Figs. 7 and 8A) and the late and tail currents increase (Figs. 7 and 8B). Late currents at -10 mV and tail currents at -90 mV evidently are equivalent measures of the progress of modification since they grow strictly proportionally in a ratio of 0.27:1.0 (Fig. 8C). At the peak, there are actually two populations of channels conducting, the modified and the unmodified ones. Since the total peak current becomes smaller during the conditioning train, the normal channels that were lost during stimulation must have contributed more current than the modified channels that were created. Qualitatively, this suggests that modified channels have a smaller conductance than normal channels. A

further analysis given in the Discussion suggests that the effective current carried by a modified channel at -10 mV may be 33% of that carried by a normal channel.

Binding and Unbinding of Veratridine

The previous paper (Sutro, 1986) shows that modification of channels occurs rapidly when normal channels are in the open state, and the modification is slowly lost at -90 mV. These processes were hypothesized to be the binding and unbinding of veratridine from its receptor. We have studied this hypothesis further.

First, we can argue that exposure to $100 \mu\text{M}$ veratridine does not spontaneously modify many channels at -90 mV. The steady state activation curve in Fig. 2A

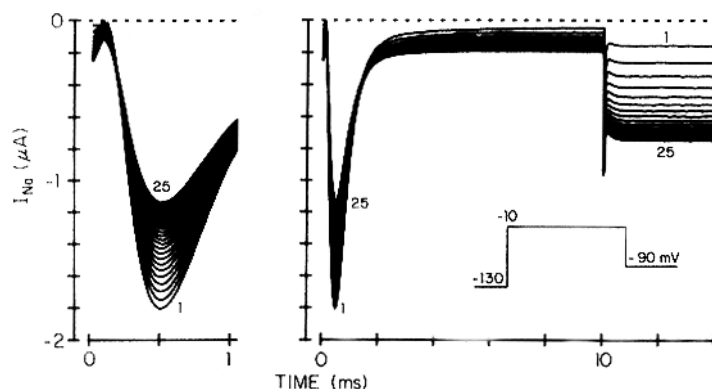


FIGURE 7. Use-dependent modification of Na channels by veratridine. A rested fiber in $100 \mu\text{M}$ veratridine was stimulated with the following pulse sequence every 272 ms for 25 repetitions: -130 mV for 50 ms, -10 mV for 10 ms, and back to -90 mV. The Na currents are plotted on two sweep speeds. Currents during the first and last pulse are labeled "1" and "25." Time zero marks the beginning of the pulse to -10 mV. In this fiber, the peak current of $1.8 \mu\text{A}$ corresponds to a current density of 3.7 mA/cm^2 .

shows that 91% of veratridine-modified channels are open at -90 mV. In each experiment, the baseline current was recorded on a chart recorder, and we inspected these records for evidence of a standing inward current at the holding potential. While there were small artifacts during the perfusion of new solutions, there was no evidence of an inward current developing as the veratridine treatment was begun or being blocked when TTX was added later. We can confidently say that if there is such a current, it is no more than 5% of the size of typical tail currents induced by our 10-pulse conditioning trains.

If the modification during a conditioning pulse is indeed *de novo* drug binding from a pool of free molecules rather than conversion of channels with prebound drug, then the initial rate of modification should be directly proportional to the free drug concentration rather than obeying a saturation function appropriate for receptors with drug already bound. Here the initial rate is determined not by suddenly applying drug to the fiber but by applying a conditioning pulse to a

rested fiber equilibrated in drug solution. To avoid uncertainty concerning the initial condition, we actually applied two pulses spaced 200 ms apart and subtracted the size of the persistent tail after the first pulse from the size after the second to obtain the rate of modification in one pulse. When this was done at different veratridine concentrations ranging from 5 to 100 μM , the initial rates showed a linear concentration dependence (Fig. 9), which supports the hypothesis that modification can be identified with binding.

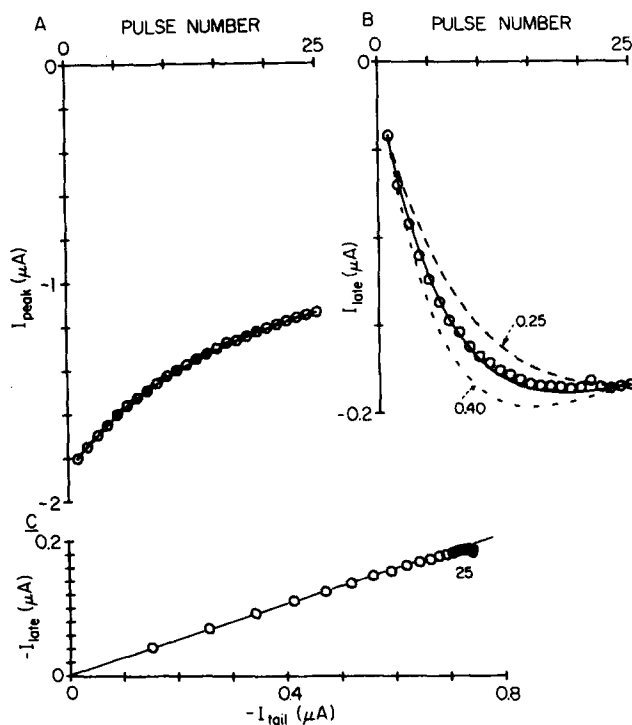


FIGURE 8. Changes of peak, late, and slow tail currents during a pulse train. Analysis of the records in Fig. 7. The measurements are shown as open circles. The lines come from a model described in the Discussion and Fig. 14 using three different values for the effective current in modified channels. (A) Peak current amplitude vs. pulse number. (B) Late current amplitude (measured just before the end of the depolarizing pulse) vs. pulse number. (C) Late current vs. slow tail current amplitude. The slope of the line is 0.27.

In the same experiments, we asked whether the rate of recovery from modification at -90 mV depends on drug concentration. A population of modified channels was induced by a conditioning train, and the subsequent decay of the persistent tail was studied kinetically. At concentrations from 5 to 100 μM , the tails decayed exponentially, with a concentration-independent time constant. This would be consistent with a pure unbinding step uncontaminated by any significant remodeling of normal channels during the decay at -90 mV.

Having described voltage-dependent gating in modified channels, we must

consider whether unbinding, like binding, depends on the gating state of the channel. Does strong hyperpolarization hasten or retard the reversion of modified channels to the normal state? For potentials between -100 and $+10$ mV, it sufficed to hold the membrane potential constant during the tail period and to record the decay of Na current (see Fig. 10C). However, for potentials so negative that many modified channels were closed, it was necessary to make brief depolarizing steps to a test potential of -74 mV, where modified channels would reopen, so we could assess how many remained. To minimize interference from the test steps, we limited them to 5 ms applied every 1.13 or 2.13 s during the 53-s experiment. Not all modified channels would reopen in 5 ms, but we assume that the maximum current achieved is a measure of the modified channels remaining. Both styles of experiments showed that unbinding follows a single-

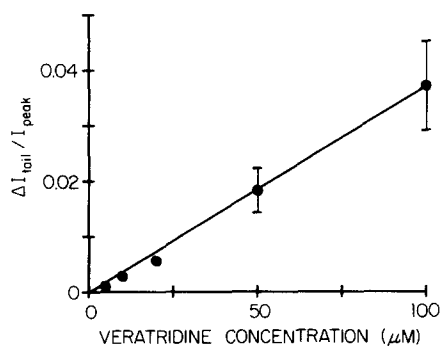


FIGURE 9. Concentration dependence of the initial rate of modification with veratridine. The pulse sequence was identical to that of Fig. 7. The slow tail amplitude, S , at -90 mV was measured after the first and second pulse, and the "rate constant" of modification was calculated as $(S_2 - S_1)/P_1$, where P_1 is the amplitude of the peak I_{Na} during the first pulse to -10 mV. Fibers were equilibrated with stepwise-increased concentrations of veratridine for several minutes before each measurement ($N = 3-13$).

exponential time course (Fig. 10, B and C), with a voltage-independent time constant at potentials more positive than -100 mV (open symbols), but at more negative potentials, the time constant for unbinding lengthens appreciably (Fig. 10A, closed symbols). The solid line shows the prediction of the following simple theory: open and shut modified channels are in rapid equilibrium with each other, governed by the equilibrium distribution determined in Fig. 2A. The assumed intrinsic time constant for veratridine unbinding is 2.9 s for open channels and 25 s for shut ones. Hence, we find that open modified channels lose drug far more readily than closed modified channels, and the rate of unbinding does depend on the state of the channel. Furthermore, unbinding from open (VO) channels is fast at even the most positive voltage tested.

Modification with DDT or Allethrin Requires Open Channels

We now ask whether two insecticides, DDT and allethrin, modify Na channels by a mechanism related to that for veratridine. Like veratridine, they retard

inactivation of Na current during a test depolarization and leave an Na tail current that persists longer than normal after the test pulse (Fig. 11, A and B). However, with either drug, the tail current decays more than two orders of magnitude faster than with veratridine, so that repetitive stimulation at 1–10 Hz induces no more modification than a single pulse. Hence, the modification

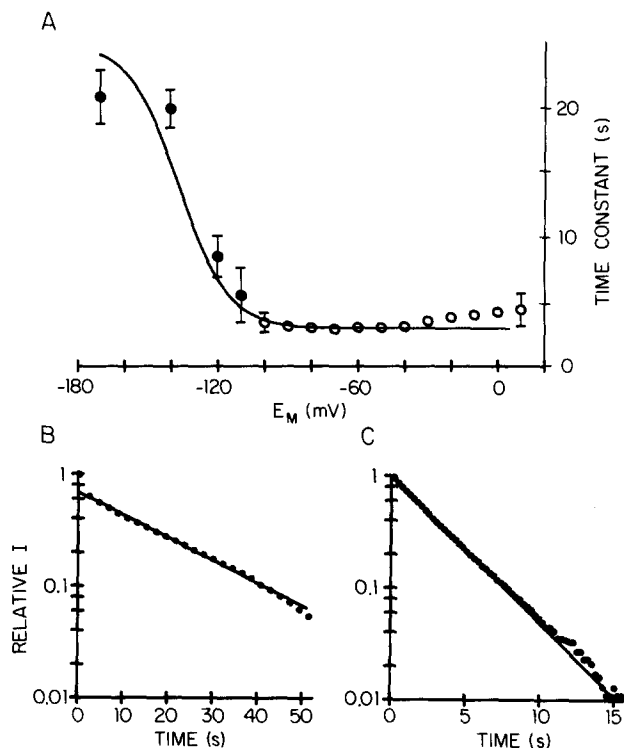


FIGURE 10. Voltage dependence of time constant for unbinding of veratridine from modified channels. (A) For the open circles ($N = 3-13$), fibers were depolarized with conditioning trains to generate modified channels and then held at the indicated potential for 50 s as the decay of current in modified channels was monitored. For the filled circles ($N = 5-12$), the unbinding time constant was determined by brief steps to -74 mV, as explained in the text. The line is the expression $[F/\tau_o + (1 - F)/\tau_c]^{-1}$, where F is the fraction of modified channels with gates open and τ_o and τ_c are assumed time constants for unbinding from open (2.9 ms) and closed channels (25 ms). (B) An example of the data at -170 mV plus the fitted exponential plotted semilogarithmically. (C) A sample tail current at -90 mV plus the fitted exponential.

induced by a test pulse is short lived. We have found that a hyperpolarizing pulse applied during the tail will speed its decay but we have not observed a reopening of the closed channels upon repolarization to -90 mV. We focus here on evidence that Na channels in the open state are needed for modification to occur.

Effect of inactivation. Two kinds of experiments show that allethrin and DDT bind better to open Na channels than to inactivated ones. First, when

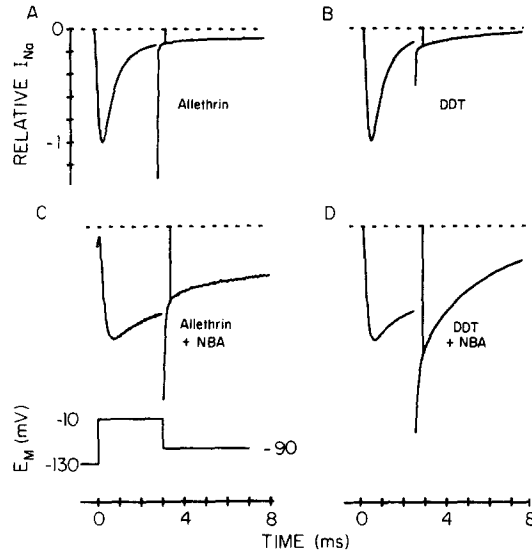


FIGURE 11. Enhancement of DDT and allethrin action by NBA. Each frame shows I_{Na} in response to a 2.5- or 3-ms test pulse to -10 mV preceded by a 50-ms prepulse to -130 mV. The tail current has two components, the rapid one (which has been truncated by sampling), presumably a combination of normal Na current and residual capacity current, and a slow, drug-induced component. A vertical line, drawn 0.4 ms after the test pulse, emphasizes the amplitude of the drug-induced slow tail current. No slow tail would be present without drug. (A) $200 \mu\text{M}$ allethrin. (B) New fiber in $250 \mu\text{M}$ DDT. (C) Fiber in A after treatment with 1 mM NBA. (D) Fiber in B after 1 mM NBA.

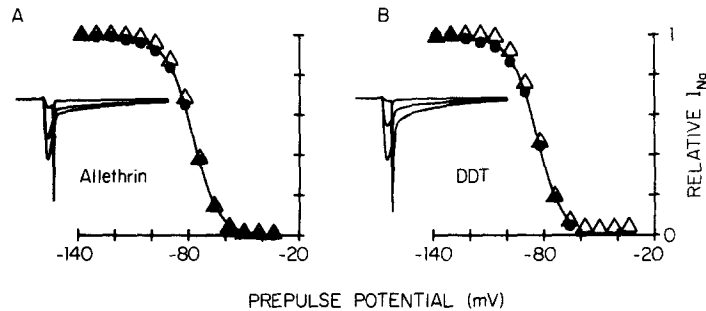


FIGURE 12. Parallel effect of inactivation caused by a conditioning prepulse on peak I_{Na} and on allethrin- and DDT-induced slow tail currents. The peak currents (filled circles) measured in a 0.75-ms test pulse to -10 mV are attenuated as the 50-ms prepulse is made more positive. The insets show sample records with prepulses to -138 , -82 , and -66 mV. The slow tail currents extrapolated to the end of the test pulse (triangles) follow the same relationship. (A) $200 \mu\text{M}$ allethrin. The curve is Eq. 1 with $E_{0.5} = -77.7$ mV and $k = 6.9$ mV. (B) $250 \mu\text{M}$ DDT. The curve is with $E_{0.5} = -83.6$ mV and $k = 6.8$ mV.

inactivation is strongly slowed by treatment with NBA, the slow tail current and hence the fraction of channels modified by drug in a single test pulse are greatly increased (Fig. 11, *C* and *D*). Second, if the number of channels opening during a test pulse is reduced by a preceding depolarizing conditioning prepulse, the number of channels appearing in the drug-induced tail is reduced as well (Fig. 12). With DDT and with allethrin, the reduction of tail current (triangles) follows

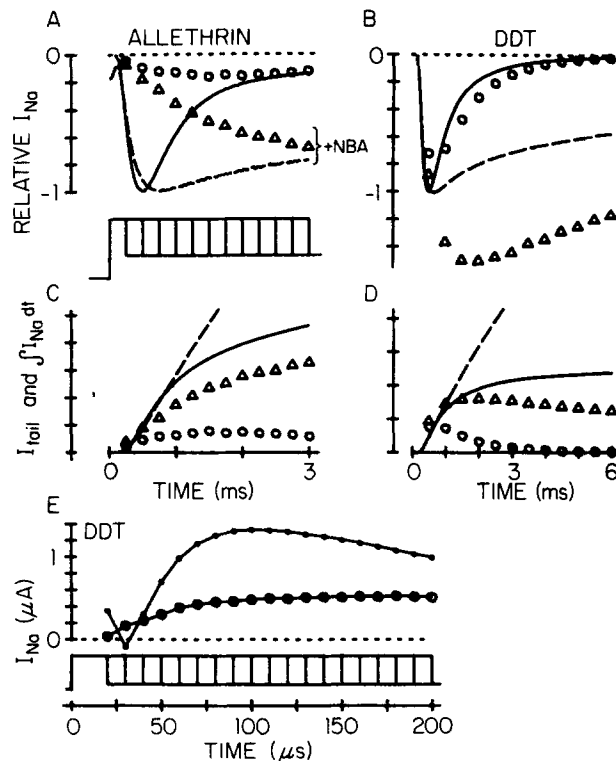


FIGURE 13. Time course of development of slow tail currents with allethrin or DDT. Same fibers and test pulses as in Fig. 11. (*A* and *B*) Comparison of I_{Na} (lines) at -10 mV with subsequent tail current amplitude (symbols). The solid line and open circles are before and the dashed line and triangles are after NBA treatment. (*C* and *D*) Comparison of running integral of I_{Na} (lines) with induced tail currents (symbols). (*E*) Rapid induction of tail currents with DDT. The filled points connected by a line are the record of I_{Na} during a pulse to $+80$ mV. The circles are amplitudes of subsequent slow tails at -90 mV with the sign inverted.

the Na inactivation curve of normal channels (circles) exactly, as if inactivated channels were completely unable to bind drug during a depolarizing test pulse.

Kinetics of modification. The development of the depolarization-induced modification can be followed by monitoring the size of slow tail currents following depolarizations of different durations. Fig. 13, *A* and *B*, plots I_{Na} during a pulse to -10 mV (solid line) and tail current sizes (circles) when the pulse was

interrupted at various times. Unlike the results with veratridine (Sutro, 1986), the tail currents did not increase monotonically as the conditioning pulse was made longer. With either allethrin or DDT, the 3-ms pulse was followed by a smaller tail than the 1.5-ms pulse. The effect was extreme with DDT, where there was virtually no tail after a 6-ms pulse. Treatment with NBA dramatically increased the size of tail currents (triangles) but did not eliminate their secondary decline, at least with DDT. The secondary decline already ensures that the time course of development of slow tail currents does not parallel the running time integral of I_{Na} , as was found for veratridine. Comparison of the integrals (lines) and tails (symbols) in Fig. 13, *C* and *D*, shows that the disagreement is extreme.

At least two explanations might be considered for the secondary decline of tail currents induced by allethrin or DDT. (*a*) The modified channels might inactivate almost as fast as normal channels, so modified channels are present in the tail period but they do not conduct. (*b*) The reaction of open channels with the insecticide may be so rapid during the pulse that it is near equilibrium. Then if drug unbinds and rebinds several times during a test pulse, the normal inactivation process will have many chances to remove unmodified, open channels from the conducting pool and the population of modified channels will be depleted. Both hypotheses have merits. With either hypothesis, the effect of NBA would be a dual one. It would make more open channels available for initial modification and remodification and it might slow the direct inactivation of modified channels.

Experiments with brief, large depolarizing pulses show that the reaction with DDT is indeed rapid. The depolarization in Fig. 13*E* was to a potential so positive (+80 mV) that I_{Na} (solid circles) was outward and reached a peak in 100 μ s. A significant tail current (open circles) appeared after 30–40 μ s of depolarization, before the outward I_{Na} was clearly resolved from the capacity current, and after 100 μ s of depolarization, the tail current was nearly maximal. Thus, the reaction takes <100 μ s at +80 mV and begins before many channels are open. At –90 mV, DDT seems to come off with a time constant of 3 ms (Fig. 11*D*). Our experiments give no independent estimate of the unbinding rate at depolarized potentials, but it would have to be faster than at –90 mV if the rapid equilibration hypothesis is the correct one.

DISCUSSION

The primary focus of this paper is to seek evidence for parallels between the actions of veratridine and some insecticides on the one hand and the better-known actions of BTX and aconitine on the other. The points we have made are: (*a*) Veratridine-modified channels open and close reversibly in an activation process whose midpoint is shifted –93 mV from that of unmodified channels. The midpoint is also sensitive to the Ca^{2+} concentration. The gating kinetics are not first order, but they lack the delay typical of activation in normal channels. (*b*) The instantaneous *I-E* relation of modified channels has a curvature indicative of voltage-dependent block by external Ca ions. Modified channels may carry less current than unmodified ones. (*c*) The initial rate of modification is proportional to the veratridine concentration, as is expected if modification is equated with binding. Recovery is independent of drug concentration. (*d*) Experiments with NBA and conditioning prepulses show that modification with allethrin and

DDT also requires open channels and does not occur with inactivated channels. Before discussing the parallels with other agents and other studies, we attempt a more careful calculation of the relative current passed by a veratridine-modified channel.

Relative Current of a Modified Channel

The experiment of Fig. 7 shows a progressive decrement of peak I_{Na} during a train of depolarizations applied in the presence of veratridine. A simple control, repeating the pulse sequence in the absence of veratridine (not shown), leads to no decrement of peak I_{Na} , so the phenomenon is not due to normal inactivation processes. Furthermore, it is not a destruction of channels, since the full peak I_{Na} returns after a 10-s period of rest. If modified channels, contributing late current, are also open at the time of the early peak, the progressive decrement means that modified channels contribute less current at -10 mV than the

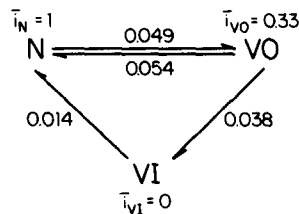


FIGURE 14. Lumped kinetic model for use-dependent modification of Na channels by veratridine. The rate constants are given as the fractional reaction during each 272-ms pulse cycle for the pulse train described in Fig. 7. The three lumped channel pools are: N, normal channels; VO, "open" modified channels; VI, inactivated modified channels. At -10 mV, a channel in each of these pools would contribute an effective current of i_N , i_{VO} , and i_{VI} , respectively. This model produced the solid lines in Fig. 8. For the dashed lines in Fig. 8, with different values of effective current, all four rate constants were readjusted to give the best fit to the change of peak currents.

unmodified channels they came from. We wish to explore this concept quantitatively.

The time courses of the decrement of peak current and the increment of late and tail current during the train can be simulated by the lumped kinetic model in Fig. 14, which generated the smooth curves in Fig. 8. It is an abbreviated form of the models in the previous paper (Sutro, 1986), where N stands for the pool of unmodified channels in all possible gating states and VO and VI stand for veratridine-modified channels in the open and the inactivated states. The three channel pools are assumed to contribute differently to the current during the pulse. The VI channels contribute no current; the VO channels contribute a steady current, $VO \cdot i_{VO}$, throughout the pulse, and the N channels contribute a transient current that reaches a peak value, $N \cdot i_N$, early in the pulse. The quantities i_{VO} and i_N are not true single channel currents, but rather they are products of single channel current at -10 mV and the probability that a channel that is in the VO or N pool is actually open at the appropriate time.

Our goal was to estimate the ratio of effective currents, i_{VO}/i_N . Various values of the ratio were tried and the four rate constants of the model were adjusted to give correct final values (after 25 pulses) of the peak and late currents, as well as a good fit to the pulse-by-pulse decrease of peak current. A good fit to late currents and peak currents resulted if VO channels (at -10 mV) had an effective current that was 33% of that of N channels at the peak (solid line). Trajectories fitted with i_{VO}/i_N ratios of 0.25 and 0.40 are drawn as dashed lines for comparison. Low values caused the late current to grow too slowly and high values caused it to grow too rapidly. If the probability that a VO channel is actually open is near 1.0 and higher than the probability that an N channel happens to be open at the peak time, then the true single channel current of a veratridine-modified channel is <33% of that of an open N channel (at -10 mV). Direct patch-clamp measurements are needed.

The rate constants for the best fit are given in Fig. 14. They are expressed per pulse cycle during the train rather than per second. Thus, in each 272-ms pulse cycle, 4.9% of the normal channels became modified (during the pulse) and 5.4% of the VO channels reverted to normal (mainly in the interval between pulses). Also during the pulse, 3.8% of the VO channels inactivated, and in the interval between pulses, 1.4% of the VI channels recovered from inactivation. These rate constants are consistent with other experiments. For example, when pulsing is turned off, they predict that the tail current would decay with a single-exponential time constant of 4.9 s.

We initially tried a linear scheme in which VI channels recovered to the VO state rather than to the N state, as in Fig. 14. This did not work, because it repopulated the VO state during the tail period, making the time course of the tail much longer, nonexponential, and markedly dependent on the intensity of the conditioning pulse train. Our lumped model might appear to violate the requirements of detailed balances as it contains one-way arrows in a cycle; however, since the VO-VI transition occurs during pulses and the VI-N transition occurs between pulses, there is no violation.

One of the more useful quantities to establish by modeling is the forward rate constant for the interaction of veratridine with open channels. Despite the simplifications of our model, we have some confidence in the idea that ~5% of the normal channels are modified per depolarizing pulse (at -10 mV), since a variety of different models gave approximately the same value. We can estimate a true rate constant for channel modification by making a couple of untested but reasonable assumptions. Suppose normal channels stay open for 0.5 ms at -10 mV and have a 50% probability of being open at the peak time. Then the rate constant of 0.05 per pulse for the whole N pool corresponds to $0.05/(0.5 \times 0.0005) = 200 \text{ s}^{-1}$ for open channels. Taking into account the drug concentration ($100 \mu\text{M}$) gives a bimolecular rate constant of $2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ for veratridine.

Similarity of Actions of Alkaloid Neurotoxins

Ours is the first description of strongly shifted activation gating in veratridine-modified Na channels, but similar shifts are well known for several other polycyclic, lipid-soluble neurotoxins. Shifts of -20 to -50 mV have been reported

after treatment with aconitine (Schmidt and Schmitt, 1974; Mozhayeva et al., 1976; Campbell, 1982; Grischenko et al., 1983), shifts of -25 to -65 mV with BTX (Khodorov et al., 1975; Khodorov and Revenko, 1979; Zubov et al., 1983), and a shift of -28 mV with grayanotoxin I (Seyama and Narahashi, 1981). The magnitude of the effects seems to depend on the cell type studied. The unusually large shift (-93 mV) with veratridine probably has not previously been noticed both because the tail current decays within seconds and because the membrane has to be hyperpolarized so strongly before the gating region is reached. As with veratridine, the modified activation gating seen with BTX or aconitine is less sigmoid in time course and slower than normal activation gating (Schmidt and Schmitt, 1974; Khodorov, 1978, 1979; Campbell, 1982; Zubov et al., 1983; Huang et al., 1982, 1984). Many studies have treated the modified gating kinetics as a single-exponential relaxation, but some recent work has noted the presence of fast and slow components such as we have seen (Zubov et al., 1983; Huang et al., 1984). The detailed kinetics are actually likely to be complex.

We found that the instantaneous I - E relation of veratridine-modified channels is N-shaped, reflecting a voltage-dependent block by external Ca ions. There are no previous reports of this with veratridine, but the phenomenon seems to be a quite general one. It has been described for Na channels of normal nodes of Ranvier (Woodhull, 1973; Hille et al., 1975), skeletal muscle (Campbell and Hille, 1976), and squid giant axons (Taylor et al., 1976). It is even more easily seen in BTX- or pyrethroid-treated channels that stay open at more negative potentials (Mozhayeva et al., 1982; Yamamoto et al., 1983). Ours may be the first demonstration that the block is relieved with extreme hyperpolarizations. No one has done a quantitative comparison in the same preparation to ask whether Ca^{2+} block differs between normal and modified channels.

From indirect evidence, we conclude that the current carried by a veratridine-modified Na channel at -10 mV is less than that of a normal channel. The decrease could be due to a combination of a lower channel conductance and a lower ionic selectivity (which would decrease the net driving force at -10 mV). All previous workers agree that macroscopic peak currents are reduced by treatments with alkaloid neurotoxins. Reduced unitary conductances for BTX-modified channels have been reported by some authors (Khodorov et al., 1981; Quandt and Narahashi, 1982), while others have found apparently normal unitary conductances (Krueger et al., 1983; Huang et al., 1984). Microscopic measurements have not been made with veratridine. This question needs to be studied with direct comparisons of normal and modified channels at the same bathing Ca^{2+} concentration and over a range of voltages. A preliminary report of ionic selectivity changes with veratridine (Naumov et al., 1979) and more complete reports with aconitine, BTX, and grayanotoxin (Mozhayeva et al., 1977; Khodorov, 1978; Huang et al., 1979; Seyama and Narahashi, 1981; Campbell, 1982) have appeared.

In summary, our experiments are consistent with Catterall's (1977, 1980) conclusion that veratridine competes with BTX, aconitine, and grayanotoxin for a common receptor. We and Sutro (1986) have identified strong qualitative similarities in the requirements for modification to occur and in the gating

properties of channels modified by veratridine and by these other neurotoxins. To complete the comparison, we would look for a more extensive analysis of ionic selectivity in veratridine-modified channels.

Action of Lipid-soluble Insecticides

Hille (1968) showed that DDT interacts transiently with normal channels only when they are in the open state and then causes them to remain open for an extra few milliseconds at the normal resting potential. This observation has been confirmed for DDT, allethrin, and their analogues by showing a reduction of tail currents by inactivating prepulses, an enhancement by agents removing inactivation, and an enhancement by activating a larger fraction of normal channels during a test pulse (Lund and Narahashi, 1981, 1982; Vijverberg et al., 1982, 1983; this paper). Lund and Narahashi (1981, 1983) have also shown that tail currents are shortened by hyperpolarization, and Vijverberg et al., (1982, 1983) have shown that modified channels inactivate relatively rapidly and completely during the depolarizing test pulse. We confirm these findings.

Despite several similarities of action, the question whether the insecticides interact with the same receptor and produce all the effects of alkaloid neurotoxins cannot yet be answered. The electrophysiologist will need to look for ionic selectivity changes and a shifted, reversible activation gating at negative potentials. The pharmacologist will need to look for direct competition in mixtures of insecticides with alkaloids.

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