Veratridine Modifies Open Sodium Channels

STEVEN BARNES and BERTIL HILLE

From the Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington 98195

ABSTRACT The state dependence of Na channel modification by the alkaloid neurotoxin veratridine was investigated with single-channel and whole-cell voltage-clamp recording in neuroblastoma cells. Several tests of whole-cell Na current behavior in the presence of veratridine supported the hypothesis that Na channels must be open in order to undergo modification by the neurotoxin. Modification was use dependent and required depolarizing pulses, the voltage dependence of production of modified channels was similar to that of normal current activation, and prepulses that caused inactivation of normal current had a parallel effect on the generation of modified current. This hypothesis was then examined directly at the single-channel level. Modified channel openings were easily distinguished from normal openings by their smaller current amplitude and longer burst times. The modification event was often seen as a sudden, dramatic reduction of current through an open Na channel and produced a somewhat flickery channel event having a mean lifetime of 1.6 s at an estimated absolute membrane potential of −45 mV (23°C). The modified channel had a slope conductance of 4 pS, which was 20–25% the size of the slope conductance of normal channels with the 300 mM NaCl pipette solution used. Most modified channel openings were initiated by depolarizing pulses, began within the first 10 ms of the depolarizing step, and were closely associated with the prior opening of single normal Na channels, which supports the hypothesis that modification occurs from the normal open state.

INTRODUCTION

Veratridine belongs to a class of lipid-soluble neurotoxins that includes batrachotoxin, aconitine, grayanotoxin, and some insecticides (Catterall, 1980; Khodorov, 1985; Hille et al., 1987). Like the other members of this group, veratridine exerts its neurotoxic action on nerve and muscle membranes by causing a persistent activation of Na channels (Ulbricht, 1969*a*; Catterall, 1980). These toxins have been called agonists or activators of Na channels.

Sutro's (1986) recent kinetic analysis of veratridine action on frog muscle concludes that Na channels must be open before veratridine can bind to its receptor. In this view, veratridine molecules would not react with normal Na channels

Address reprint requests to Dr. Bertil Hille, Dept. of Physiology and Biophysics, SJ-40, University of Washington School of Medicine, Seattle, WA 98195.

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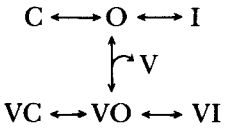
when they are resting or inactivated, but only when they are open, as in the following reaction scheme:



(Scheme I)

Here the upper line represents the standard, voltage-gated transitions of a normal channel from closed, resting states (C) to an open state (O) and inactivated states (I) during a depolarization. Veratridine (V) is shown reacting only with the open state to give a persistent, veratridine-modified open state (VO), which, with appropriate voltage protocols, can be induced to close (VC) or to inactivate (VI) without losing the bound neurotoxin.

Sutro's evidence for Scheme I came from a dissection of macroscopic Na currents into a transient component presumed to come from normal channels and a persistent component from modified channels. He showed that the rate of induction of the persistent, modified component by a depolarizing pulse was proportional to the number of normal channels opened, as would be expected for a simple precursor-product relation between the O and VO states. A second study of macroscopic currents in the same preparation continued the kinetic analysis of modified Na channels (Leibowitz et al., 1986). It showed that the cumulative effects of repetitive stimulation could be quantitatively accounted for by Scheme I if ~5% of the normal channels opening in each depolarizing pulse became modified (100 μM veratridine), if the effective current through a modified channel was reduced to one-third that in a normal channel at −10 mV, and if the lifetime of the modified state was on the order of seconds. These ideas were then extended to other veratrum alkaloids (Leibowitz et al., 1987).

We wanted to test these assumptions at the single-channel level and used neuroblastoma cells, where single Na channel currents have been characterized in detail (Aldrich et al., 1983; Aldrich and Stevens, 1987). Since the macroscopic effects of veratridine on these cells were not known, we have done whole-cell as well as single-channel recording. A preliminary report has appeared (Barnes and Hille, 1987).

METHODS

N18 neuroblastoma cells were maintained in culture using standard techniques (Catterall, 1986; Gonoi and Hille, 1987) with the help of Dr. William Catterall's group in the Pharmacology Department at this university. Cells used for recording were grown for 1–7 d in Dulbecco's modified essential medium with 5% fetal calf serum at 37°C and 10% CO_2. 1–5 h before recording, they were washed in mammalian Ringer (150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM Na HEPES, buffered to pH 7.4) and maintained at room temperature (21–23°C). Under these conditions, many cells take up a rounded morphology suitable for whole-cell voltage-clamping.

Whole-Cell and On-Cell Single-Channel Recording

Whole-cell pipettes were pulled from glass hematocrit tubes (VWR, Seattle, WA, or Curtin-Matheson Scientific Inc., Houston, TX) in two steps on a vertical pipette puller,

coated with Sylgard 182 (Dow Corning, Midland, MI) to within 100 μm of the tip, and lightly fire-polished. When the pipettes were filled with intracellular solution (90 mM CsF, 60 mM CsCl, 10 mM NaF), the tip resistances measured in the bath were between 500 and 1,000 kΩ. Cs was used as the major intracellular cation to reduce currents in K channels. Electrodes for single-channel recording were pulled from Corning 7052 glass (Garner Glass Co., Claremont, CA) in a similar manner. They were coated, fire-polished, and filled with a hypertonic, low-Ca solution chosen to increase the size of single Na channel currents (300 mM NaCl, 5 mM KCl, 300 μM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM Na HEPES, pH 7.4). They had tip resistances near 10 MΩ measured in the bath. Seal resistances ranged from 10 to 100 GΩ.

Electrode tip potentials were measured against a ceramic junction, saturated KCl, reference electrode (29402, Beckman Instruments, Inc., Fullerton, CA). Whole-cell pipettes were 5.1 ± 0.5 mV (SD, $n = 5$) negative when the voltage-clamp current was zeroed with the pipette in the standard bath filled with Ringer. Therefore, the recorded whole-cell membrane potentials were corrected by subtracting 5 mV. Potentials for on-cell patches are given relative to the unknown resting potential of the cell, using, for example, the notation RP−90 mV to mean a 90-mV hyperpolarization from the resting potential. Because of the uncertainty of the absolute potential, no junction potential correction was made. (It would require subtracting an additional 2 mV.)

The EPC-7 patch clamp (List Electronic, Darmstadt, Federal Republic of Germany) was used with capacitance and series-resistance compensation to record membrane current. Further analog capacitance compensation and leak subtraction were made before the signal was low-pass-filtered at 5 kHz (−3 dB point, four-pole Bessel), digitized with either a modified Sony PCM-501 pulse-code modulator (Unitrade, Philadelphia, PA) or a LabMaster A/D board (Scientific Solutions, Solon, OH) in an IBM AT computer (IBM, Boca Raton, FL), and then stored on video tape or computer disk. Slow time scale playback was made onto a chart recorder or by redigitization into the computer, while high temporal resolution playback was made by direct digital transfer of the PCM-501 16-bit signal to the IBM AT computer utilizing the hardware modification of Bezanilla (1985) and the BASIC-Fastlab Programming System (Indec Systems, Sunnyvale, CA). The combination of analog and/or digital filtering used in drawing the figures is summarized in the figure legends by the effective half-power (−3 dB) frequency, f_h, of the record. Experiments were performed at room temperature (21–23°C) or with slight cooling (19°C).

Analytical

Some measurements of voltage dependence have been fitted with a Boltzmann function: $1 + \exp[(E - E_{0.5})/k]^{-1}$, where E is the membrane potential and $E_{0.5}$ and k are referred to as the midpoint potential and the slope factor. Midpoint potentials, slope factors, and other mean values are reported in the text as means ± SD.

The brief openings of unmodified, single Na channels were scored by the 50% amplitude threshold criterion (Colquhoun and Sigworth, 1983). The amplitude of well-resolved openings was measured, and any point rising to 50% of this value was considered to represent an opening. As part of a test of Scheme I discussed later, we wanted to describe the distribution function of the number of openings in various subsets of sweeps. A few of the details of the calculations are given here. If openings are independent and there are o_d detected openings in s sweeps, the distribution of openings follows a binomial distribution for N channels opening with a probability of $o_d/(sN)$ per sweep (Colquhoun and Hawkes, 1983). If, because of the finite frequency response, only a fraction, f_d, of the actual openings are detected, the distribution of actual openings, o_a, fol-

lows another binomial distribution for N channels opening with a probability $o_d/(sf_dN)$. If, as in Scheme I, each actual open channel can bind veratridine with a probability p_v, then of the subsets of sweeps with exactly n normal openings, a fraction $(1 - p_v)^n$ will not include a reaction with veratridine. With these considerations, one can predict the distribution of actual openings for sweeps that do or do not yield a reaction with veratridine. These distributions need to be corrected finally for the fact that only f_d of the actual openings will be detected. It is easy to show for the sweeps with exactly n openings that a fraction $(1 - f_d)^n$ will mistakenly be thought to have fewer openings. It takes more algebra to write down exactly how many openings will be detected in these sweeps for which at least one opening is missed. We state the result as an iterative computer algorithm that repeatedly moves a fraction $(1 - f_d)$ of the sweeps with i openings into the category with $(i - 1)$ openings. This transfer is done inside a loop with i stepping from n down to k. This inner loop is nested inside another loop with k going from 1 to n.

Application of Veratridine

Veratridine (Sigma Chemical Co., St. Louis, MO) at a final concentration of 100–166 μM was applied to the bath during whole-cell recording. Modification of channels was usually apparent within 20 s. Washout of the drug was not attempted. For the single-channel experiments, veratridine was added to the hypertonic pipette solution (high Na^+, low Ca^{2+}) at a concentration of 100–250 μM. Some modified channels were apparent as soon as single-channel activity could be seen.

RESULTS

This study had three primary goals. The first was to repeat in neuroblastoma cells some of the experiments with macroscopic currents that had led to the hypothesis of Scheme I in frog skeletal muscle, concentrating particularly on interconversions between the normal and modified channel populations. This revealed a qualitative similarity in the effects of veratridine on the two preparations. The second goal was to identify and characterize single-channel currents through veratridine-modified channels. The third goal was to attempt to observe the actual modification event, which according to Scheme I would consist of the opening of a normal channel followed by a conversion into a channel of lower conductance and longer lifetime.

Macroscopic Currents Show Use-dependent Modification

As in other cell types, we found that modification of neuroblastoma Na currents by veratridine was enhanced by repetitive depolarizations. Fig. 1 *A* shows superimposed Na current traces in a cell during a train of depolarizing pulses. Veratridine (166 μM) had been applied to the bath 5 min earlier, and the cell had already been depolarized in veratridine by several pulse trains separated by rest periods before this recording. In the first pulse of this train (labeled "1"), Na current activated to a peak and then inactivated in nearly normal fashion, except that inactivation was incomplete—the Na current did not return fully to the baseline. After the pulse, there was a standing inward tail current representing a population of veratridine-modified Na channels that did not close at −85 mV. With repeated pulsing at 6 Hz, the transient peak shrank and the noninactivating current and the standing tail current grew. We interpret this as a progressive

conversion of the unmodified Na channels that make the transient peak current at −5 mV into modified ones that remain open both at −5 and at −85 mV.

Once the train of depolarizing pulses is turned off, the signs of modification persist for only a few seconds. During this time, the tail current decays away and the ability to evoke large transient Na currents returns, despite the continued presence of veratridine. Fig. 1 *B* is a slow record of a similar experiment, heavily filtered to emphasize the growth of the tail current during a 10-Hz train of depolarizations like that of Fig. 1 *A*, and its subsequent decay during a period of rest. All of these currents could be blocked by tetrodotoxin (not shown). The tail current decayed exponentially during the rest period with a 0.94-s time con-

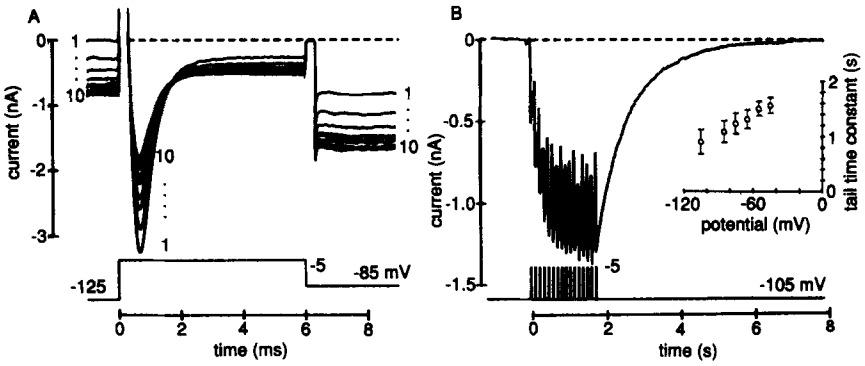


FIGURE 1. Induction and decay of veratridine-modified, open Na channels. *(A)* 10 identical depolarizing steps were applied at 6 Hz and the resulting whole-cell currents, labeled "1" through "10," are superimposed on a fast time scale. A 100-ms hyperpolarization to −125 mV preceded each 6-ms test pulse to −5 mV. 166 μM veratridine in the bath. T = 22°C; f_h = 5 kHz. *(B)* 16 5-ms depolarizations were applied at 10 Hz and the whole-cell currents are drawn on a continuous, slow time base. No hyperpolarizing prepulse was used. 166 μM veratridine in the bath. T = 23°C; f_h = 30 Hz. *(Inset)* Voltage dependence of the decay time constant of the tail current after a train of pulses. Points are means ± SD, n = 4. T = 22–23°C.

stant. In most experiments with short trains of depolarizing pulses, at least 95% of the relaxation is described by a single exponential when the tail is measured at potentials between −90 and −50 mV. The time constant of decay proved to be weakly dependent on the membrane holding potential (inset to Fig. 1 *B*), slowing with increasing depolarization approximately e-fold/110 mV. In this voltage range, we consider the decay of the persistent current to represent dissociation of veratridine from the Na channel, restoring the channel to its unmodified state. Thus, in agreement with Leibowitz et al. (1986), we believe that the complex between the neurotoxin and an open channel typically has a slightly voltage-dependent lifetime on the order of 1 s. Particularly when long

trains of depolarizing pulses were given, the relaxation of the tail current was better described by two exponentials with closely spaced time constants. The slower time constant was on the order of 2–4 s.

The modification with veratridine is quickly reversible in the sense that after the tail current has decayed away, the induction of tail current at the expense of peak current can be repeated by applying another train of depolarizing pulses. However, not all Na channels return to normal. In the first pulse train after veratridine is applied, the peak Na current starts out about as large as in the control, but from then on the initial Na current may be 10–30% smaller, as if a small fraction of the channels remained in a modified, but not open, condition in 166 μM veratridine.

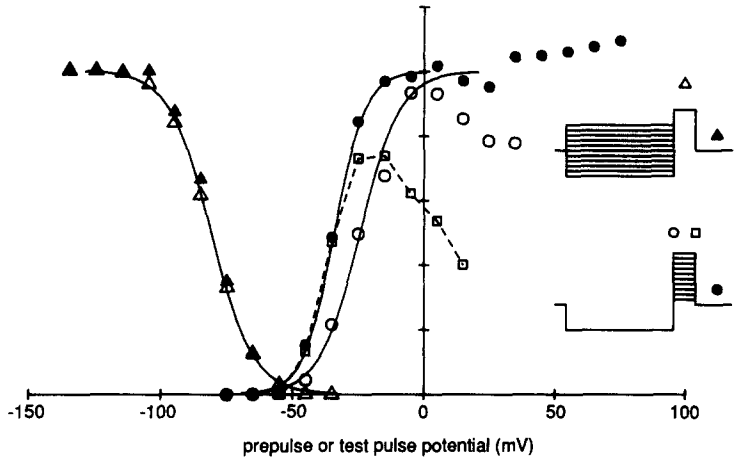


FIGURE 2. Influence of Na channel activation and inactivation on induction of whole-cell tail current. The open circles and triangles plot the relative peak transient conductance during a depolarizing pulse and the filled symbols plot the relative tail current 5 ms after a return to the holding potential of −90 mV in the same sweep. The open squares and dashed line plot integrals of Na conductance during the test pulse. The voltage of the 100-ms prepulse was varied to get an inactivation series (triangles), and that of the 6-ms test pulse was varied to get an activation series (circles and squares). The smooth curves are Boltzmann fits with midpoints and slope factors of −81 and 8, −34.1 and −5.8, −24.3 and −7.5 mV. 166 μM veratridine in the bath. T = 22°C.

A kinetic test for a precursor-product relation between the O and VO states of Na channels in Scheme I is to measure the size of the persistent tail current following single depolarizing pulses that open different numbers of Na channels (Hille, 1968; Sutro, 1986). There should be a direct proportionality. The number of normal channels opened is easily varied in two ways. The first is to use conditioning prepulses that inactivate some of the channels before the depolarizing test pulse. The triangles in Fig. 2 are the results of such an experiment on a cell exposed to 166 μM veratridine. The open triangles show how the prepulse voltage affects the peak transient Na current at a constant test potential of −15 mV, a classic inactivation curve. The persistent tail current (filled trian-

gles), measured 5 ms after the same depolarizing test pulse ended, follows the same function, as would be expected for a precursor-product relation.

The second way to vary the number of normal channels opened is by using test pulses of different amplitudes in the voltage range where activation of normal Na channels is steeply voltage dependent. The open circles in Fig. 2 show the voltage dependence of the peak Na conductance (chord conductance) in the same cell as in Fig. 1 *A*. In the voltage range from −55 to −5 mV, the persistent tail currents (filled circles) following each test pulse have an approximately parallel voltage dependence that is, however, shifted 9 mV to the left of the peak activation curve. As Sutro (1986) argued, it is actually not the peak number of normal channels open but rather the time integral that should predict how many channels become modified in Scheme I. Normalized integrals of the Na conductance in these same sweeps, shown as open squares connected with a dashed line, rise steeply with depolarization but then reach a maximum near −20 mV and fall off with further depolarization. This property of normal Na currents agrees well with findings in crayfish axons and frog muscle (Bean, 1981; Sutro, 1986). As Sutro (1986) also found, the integrals describe the voltage dependence of modified-channel induction well at small depolarizations but then fall below the induction curve at large depolarizations. Thus, this experiment again supports Scheme I, if we accept that the effective rate constant for the O-to-VO transition increases with depolarization in a fairly voltage-dependent manner.

These kinetic experiments also agree with the hypothesis that many Na channels remain unmodified when a cell is held at a large negative holding potential in veratridine and is stimulated only infrequently. Consider the activation and inactivation measurements of peak transient currents (Fig. 2), which have been fitted with smooth curves derived from the Boltzmann relation. In veratridine-treated cells, the midpoint and slope factors of the curves were −23.7 ± 3.5 and 7.2 ± 0.3 mV (n = 5) for activation and −81.3 ± 5.0 and 8.5 ± 1.6 mV (n = 6) for inactivation, values indistinguishable from those for untreated cells, which were −23.3 ± 4.9 and 6.5 ± 0.5 mV (n = 4) for activation and −81.9 ± 3.0 (n = 8) and 8.5 ± 1.8 mV (n = 5) for inactivation. We can say at least that a kinetically normal, transient Na current remains in the comparatively high 166 μM veratridine concentration used here.

Modified Single Channels Have the Expected Properties

We turn now from macroscopic recording to on-cell patch recording of single channels. Because of the low resting potentials of our cells (see later), Na channels were fully inactivated. In order to open Na channels with depolarizing steps, we had to hold the on-cell membrane patches hyperpolarized at RP−30 to RP−100 mV. Brief openings then appeared during the first few milliseconds of a depolarizing voltage step. Fig. 3 *A* shows seven sweeps filtered at 3.9 kHz and exhibiting normal-looking Na channel openings as downward, 1.8-pA events. A cumulative open-time histogram (Fig. 3 *B*) of the first 208 openings in this experiment gave a mean Na channel open time of 253 μs (19°C). Although this patch had been treated with 250 μM veratridine, we judge these events to

be openings of normal (unmodified) channels since they do not differ from openings seen in control patches without veratridine.

Many sweeps in the same experiment induced another class of Na channel openings (Fig. 3 *C*). These were much smaller, long-lasting bursts of events best seen at a slow sweep speed with heavy filtering of the record. The trace of Fig. 3 *C* shows four such bursts of −0.28 pA mean event amplitude. Each time one

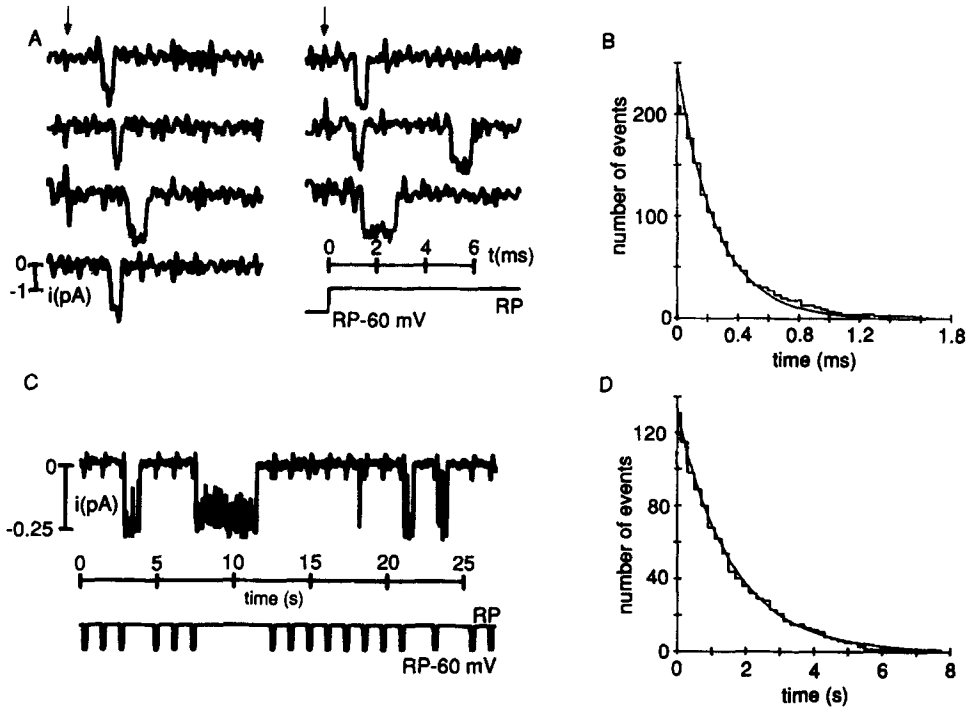


FIGURE 3. Openings of normal and modified Na channels in veratridine-treated on-cell patches. Openings are elicited by depolarizing to the resting potential (0 mV pipette potential) after a 200-ms, 60-mV hyperpolarization. *(A)* Selected brief, normal-looking openings. 250 μM veratridine in the pipette. T = 19°C; f_h = 3.3 kHz. Arrows mark the beginning of the depolarizing step from RP−60 mV to RP. *(B)* Cumulative histogram of open dwell time for the normal openings, including those in *A*. The curve is an exponential with a time constant of 253 μs. *(C)* Long modified openings occurring during the same experiment. f_h = 30 Hz. *(D)* Cumulative histogram of burst times for modified openings at RP−40 mV in a different cell. The curve is an exponential with a time constant of 1.6 s. 250 μM veratridine in the pipette. T = 23°C.

occurred, we delayed the hyperpolarizing potential step until the events terminated. Cumulative histograms of burst durations are adequately described by single exponentials. That of Fig. 3 *D* has a decay time constant of 1.6 s (23°C), which would be consistent with the mean macroscopic tail decay time near −45 mV absolute potential (inset to Fig. 1 *B*). We were unsure of the absolute potential in on-cell patch recordings, but the ensemble-average kinetics of normal

openings and the resting potentials measured in the whole-cell, macroscopic recordings consistently indicated rest potentials between −25 and −5 mV. If the cell from which the histogram was measured rested near −5 mV, then the additional 40-mV hyperpolarization applied during the measurement would be consistent with an absolute potential of −45 mV.

Another feature of the modified channel is the small current it passes. The current-voltage relation for modified events in a different cell (filled circles, Fig. 4) gave a slope conductance of only 4 pS. (Although we biased our estimate of the single-channel current to ignore the many transitions toward zero current in these flickery openings, the 4-pS value may slightly underestimate the actual open-channel conductance.) At five voltages in that experiment, amplitudes of normal openings (open circles) could be determined as well. The slope of the regression line through these points was 16 pS and, for three experiments, aver-

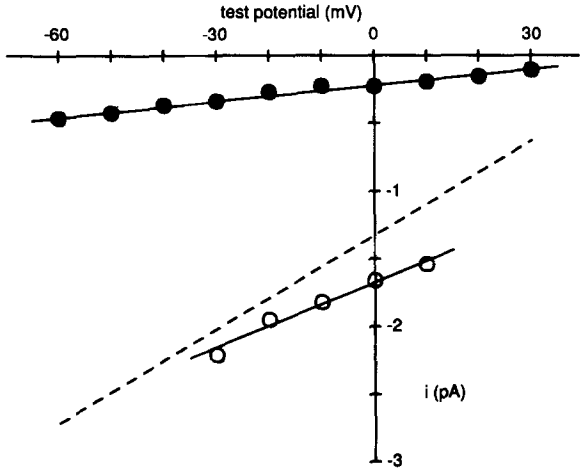


FIGURE 4. Current-voltage relations of normal and modified channels. The points are averages of many normal (open circles) and modified (filled circles) openings from a single on-cell patch. The solid lines are regression lines with slopes and voltage intercepts of 16 pS and RP+56 mV and 4 pS and RP+105 mV. The dashes are the regression line for modified channels scaled up sixfold. Voltages are relative to rest. 250 μM veratridine and hypertonic Ringer in the pipette. T = 18°C.

aged 19 ± 3 pS. While the typical slope conductance of modified events was one quarter of that for normal events, the current at RP−30 mV was more than sixfold smaller because the regression lines extrapolated to different x axis intercepts. The difference is apparent in comparing the open circles from normal channels with the dashed line, which is a scaled-up (six times) version of the modified channel i-V curve. This difference is in the direction expected if modified channels have a lower Na selectivity (Naumov et al., 1979; Garber and Miller, 1987; Leibowitz et al., 1987), but linear extrapolation is not a reliable method for determining true reversal potentials. Not shown in Fig. 3 are rare bursts whose events had a different current magnitude of one-half or four-thirds the size of the usual modified events. While they clearly existed in records from two of five patches in which current amplitudes were looked at carefully, we have seen too few of them to discuss their conductance, selectivity, or kinetics.

We refer to the openings in Fig. 3 *C* as bursts because they seem to have many brief closings, better seen in expanded records. Fig. 5 shows a short (585 ms) burst originally recorded on video tape at 5 kHz bandwidth, but replayed and filtered at 1,000, 500, and 250 Hz. In the 1,000-Hz record, there are at least 96 "closing" spikes, transitions in which the current trace fell briefly to <50% of the modified open level. If these are poorly resolved full closings, the channel openings within a burst typically last <6.4 ms and the closings last <300 μs. Twice during this short burst there were also longer (9.5 and 8.5 ms) apparent

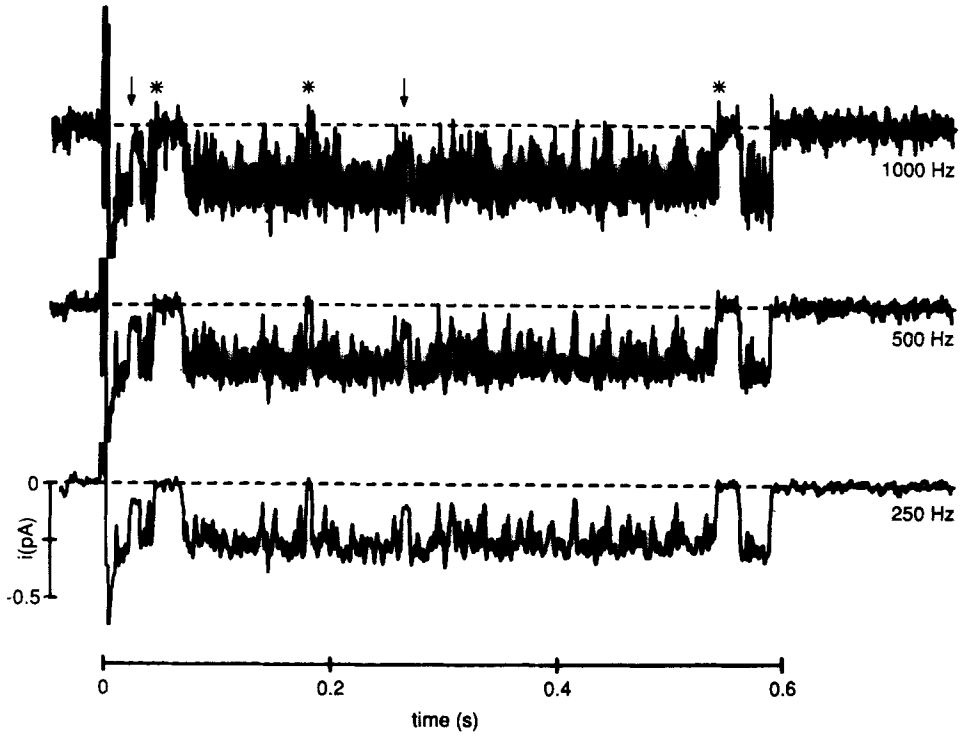


FIGURE 5. Structure of a short (585 ms) burst of modified openings at three different filter settings and digitizing frequencies. From top to bottom, f_h = 1,000, 500, and 250 Hz. The burst was induced by turning off a hyperpolarization to RP−60 mV. Two closings to a substate are marked with arrows and three full closings are marked with asterisks. 100 μM veratridine in the pipette. T = 22°C.

partial closures (arrows) to low-current substates. In addition, there were three obvious full closures of 26.5, 2, and 22.5 ms in the 585-ms trace. The same behavior was seen in another burst examined like this.

It is conceivable that this substate and even the typical 4-pS modified state are artifacts—unresolved high-frequency gating between the 15–20-pS unmodified level and zero. We do not consider this likely, however. During the substate, the current noise is no larger than it is after the burst, when there is no gating. During the 4-pS state, the noise is larger but, as less and less filtering is used, it

all seems to reflect gating toward zero current and there is no tendency for spikes of current to grow toward the 1.8-pA current level of an unmodified channel.

The Modification Event at the Single-Channel Level

As was seen from the tail currents at the macroscopic level, induction of single modified events was use dependent. Modified channels opened infrequently when the cell was held continuously at one membrane potential, whether this

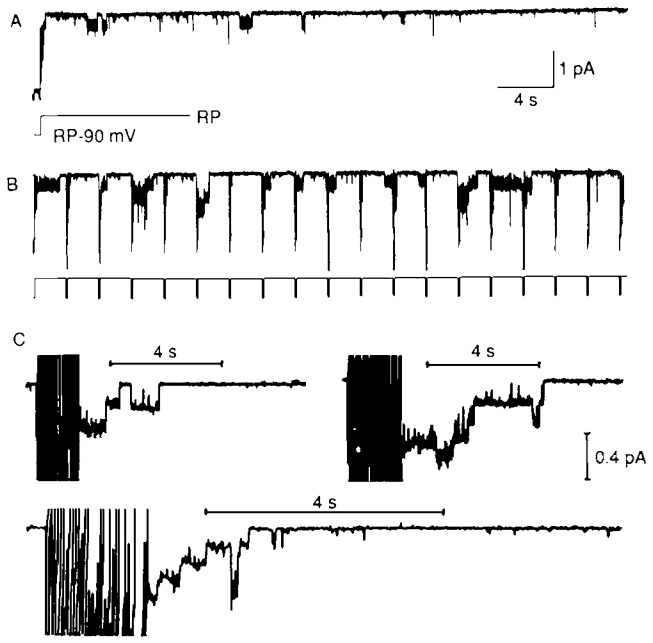


FIGURE 6. Induction of modified openings by depolarizing steps. *(A)* A single long depolarization to RP+0 mV. The current trace is mostly quiet. 250 μM veratridine in pipette. T = 19°C; f_h = 300 Hz. *(B)* A repeated pattern of 200-ms hyperpolarizations followed by 800-ms depolarizations to 0 mV induced modified openings repeatedly. Same patch and time scale as in *A*. *(C)* Another patch shows a build-up of multiple modified openings induced by rapid trains of steps to RP+0 mV from RP+80 mV. 250 μM veratridine in the pipette. T = 22°C; f_h = 50 Hz.

was a relatively negative or a depolarized level. Voltage steps from negative to more positive levels induced modified events, much as they also favored normal openings. Fig. 6 *A* shows a single depolarizing step to RP+0 mV from a holding level of RP−90 mV. Soon after the step, modified channels closed, the current trace became relatively quiet, and, as the depolarization was maintained, there were only infrequent, single modified openings. In contrast, when the cell was repeatedly hyperpolarized to RP−90 mV (Fig. 6 *B*), each subsequent depolarization induced one or more modified openings. In patches with fewer channels

or when the prepulse potential was less negative (Fig. 3 *B*), the probability of seeing a modified opening was much lower than in Fig. 6 *B*, but even in such cases, if a rapid train of pulses was delivered (Fig. 6 *C*, from a different cell), a build-up of modified events occurred, and when the train was stopped, they decayed away.

If Scheme I were a complete kinetic description, then every time a channel binds veratridine, one should see a normal opening turning into a modified opening (and every time the drug dissociates from the channel, one should see a modified opening turning into a normal opening). We wanted to examine the first prediction in detail. Ideally, a study of the events during modification

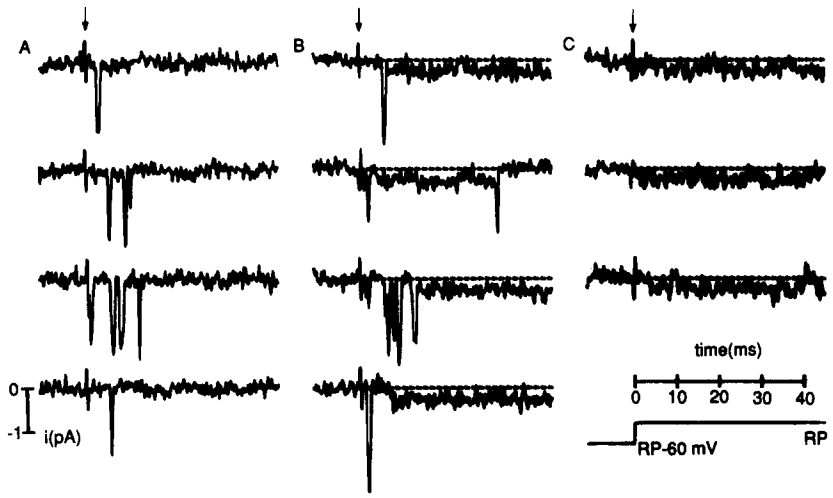


FIGURE 7. Selected nonblank sweeps measured with the voltage protocol of Fig. 3 *C*, showing the last 10 ms of the 200-ms hyperpolarization (to RP−60 mV) and the first 45 ms of the period at RP+0 mV. Same experiment as for Table I and Fig. 3, *A–C*. Arrows mark the beginning of the depolarizing step from RP−60 mV to RP. *(A)* 4 of the 275 sweeps with normal openings but no modified openings. *(B)* 4 of the 70 sweeps with both kinds of openings. *(C)* Three of the four sweeps with modified openings but no detected normal opening. T = 19°C; f_h = 1,100 Hz.

should be done on patches with only one channel; however, since such preparations are not easily found, we were forced to analyze multichannel patches by statistical methods. The analysis was done on several hundred consecutive depolarizations (sweeps) in three on-cell patches. For purposes of illustration, we describe in detail the results with 468 consecutive sweeps in the experiment shown in Fig. 3, *A–C*.

Selected sweeps from this experiment are shown in Fig. 7. Column *A* shows a few sweeps with normal openings alone, including some with several normal openings, and column *B* shows four sweeps with both normal and modified openings. Each sweep was examined at several combinations of sweep speed and

filtering to count the openings. We examined only the first 45 ms of the record for normal openings but considered modified events that appeared any time during the depolarizing step. Modified events were easily recognized by eye in strongly filtered records. Between zero and five normal openings were found in each sweep. Since each channel usually opens only once before inactivating in neuroblastoma cells (Aldrich et al., 1983; Aldrich and Stevens, 1987; Gonoi and Hille, 1987), this patch probably contains at least five channels (see later). The distributions of the number of channel openings are summarized in Table I.

We consider the test of Scheme I in stages. First, we consider qualitative consistency with the data. Then we see if we can use the hypothesized precursor-product relationship between the O and VO states of the scheme to predict the sweep-by-sweep association of normal and modified openings summarized in Table I. Then we test the temporal relationship of modified to normal openings. Finally, we consider possible deviations from the scheme.

TABLE I

Numbers of Openings per Sweep

Normal openings	All sweeps	With modified openings	Without modified openings
0	123	4	119
1	168	29	139
2	96	16	80
3	42	12	30
4	29	9	20
5	10	4	6
Total sweeps	468	74	394
Total openings	652	153	499
Openings/sweeps	1.39	2.07	1.27

From 468 consecutive sweeps with 250 μM veratridine. T = 19°C.

The association of normal and modified openings was qualitatively strong. Of the 74 sweeps that had modified openings, 70 of them also had normal openings (Table I), at least one of which always preceded the modified event. Fig. 7 *B* shows selected traces from this group, which will be considered again later. On average, the sweeps with modified events were found to have more normal openings than the sweeps without modified events, 2.07 vs. 1.27, as well as a far higher probability of having at least one normal opening, 0.95 vs. 0.74 (Table I). Such an enrichment is expected from Scheme I, since the more normal openings there are, the more likely is the occurrence of a modification.

What about the four sweeps with modified openings but no detected normal opening (Fig. 7 *C*)? These may indeed be sweeps without normal openings, but it is also expected with analog and digital filtering that the briefest normal openings will be missed when using the 50% amplitude criterion (reviewed by Blatz and Magleby, 1986). The probability of missing a normal opening can be estimated. An empirical test showed that with the highest effective frequency response we used (~3.9 kHz), we would have missed events with open times

shorter than 55 μs. Since the mean open time for normal Na channels in this patch was 253 μs (Fig. 3 *B*), a fraction, exp(−55/253), or only 81% of the openings, should have been detected and 19% missed.

We now have almost enough quantitative information to reconstruct the complete distribution of openings summarized in Table I, assuming that normal openings are not correlated with each other. We know that during 468 depolarizations, there were 652 detected normal openings and 81 modified openings, that the probability of missing normal events is 0.19, and that there are at least five channels. The actual number of channels is unknown. Assuming that the normal openings are not correlated with each other, their distribution is readily predicted from the binomial theorem, the only free parameter being the choice of the number of channels in the membrane. Fig. 8 *A* shows the prediction for 10 channels (solid line) compared with the observations (open circles). The number of channels did not make much difference as long as a number between 7 and 13 was used.

Now consider the partitioning of this distribution function into subsets with and without modified openings. Assuming that modification events follow normal openings with a fixed probability (and are independent of each other) and then correcting for missed events, we can predict the distribution functions shown by the dashed lines in Fig. 8 *A*. They resemble the actual observations, shown by the filled circles (modified events) and open triangles (no modified events). In close agreement with Table I, they predict means of 2.01 and 1.27 detected openings per sweep, and they predict that 3.9 sweeps with modified events would appear to have no normal openings (because of the 19% detection error). Thus, Scheme I gives a good account of these observations.

Results with two other membrane patches analyzed in the same way are shown in Fig. 8, *B* and *C*. As was found with the first patch, sweeps with modified openings have more normal openings than those without modified openings, and the theory based on Scheme I gives an adequate description of this enrichment. The observed numbers of normal openings in these categories are 2.22 and 1.51 per sweep for the second patch and 2.89 and 2.71 for the third. The corresponding means predicted from the theory are 2.23 and 1.49 for the second patch, and 3.01 and 2.69 for the third.

Another test of Scheme I looks for the temporal relationship between opening of normal and modified channels and makes use of ensemble averages of two subsets of sweeps. One includes all sweeps without modified openings, and the other includes sweeps with modified openings. The first ensemble average (curve 1 in Fig. 9, top) resembles the transient macroscopic Na current of an untreated cell, emphasizing again the presence of normal Na channels in cells treated with 166 μM veratridine. The other ensemble average (curve 2) has a transient component followed by a steady current. The presence of a large transient component shows again that, on average, normal Na channels continue to open early in the sweeps that contain modified channels. The flatness of the steady component means that modified openings also tend to begin early in the sweep. The opening time of modified events could be determined if we could subtract from curve 2 the component of current arising from normal channels. This compo-

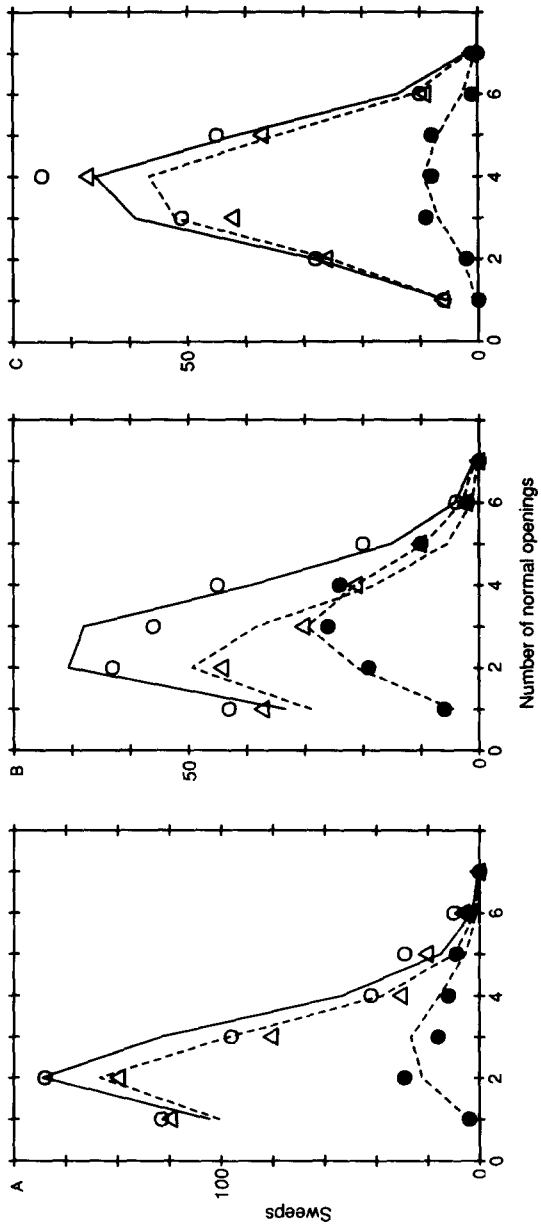


FIGURE 8. Distribution functions for the number of detected normal openings per sweep in three patches. *(A)* The experiment of Fig. 7. Symbols are the observations compiled in Table I: open circles: all sweeps; triangles: sweeps without modified openings; filled circles: sweeps with modified events. Solid line: distribution function for all sweeps predicted from the binomial distribution for 10 channels with a probability o_a = 0.171 of opening per sweep and a probability f_d = 0.81 of being detected when open. Dashed lines: distribution functions for sweeps with and without modified openings predicted on the assumption that each normal opening has a probability p_v = 0.101 of inducing a modified opening. The dashed lines were calculated in two steps (see Methods). The binomial distribution of normal channels (detected and not detected) was used to calculate how many sweeps of each category would induce modified openings. These new distributions were then adjusted to account for the probability of missing an opening. *(B)* Similar analysis for 231 sweeps in another patch, using a binomial distribution for 11 channels with o_a = 0.215 of opening, f_d = 0.75 of being detected, and p_v = 0.203 of inducing a modification. 250 μM veratridine in the pipette. T = 22°C. *(C)* Another patch with 216 sweeps, assuming six channels, o_a = 0.608 of opening, f_d = 0.75 of being detected, and p_v = 0.039 of inducing a modification. 100 μM veratridine in the pipette. T = 23°C.

nent probably would have a time course like curve 1, but scaled up to take into account the larger number of normal openings per sweep in the records contributing to curve 2. The factor would be $\sim 2.07/1.27 = 1.63$. The result of such a subtraction, shown as dots in Fig. 9 (bottom), should represent the contribution of modified channels, and, because their lifetime is much longer than these traces, would be identical to a cumulative latency histogram for modified openings (latency between the start of the depolarization and the start of the opening). According to Scheme I, this latency histogram should have the same time course as the integral of the normal Na current (solid line), which it does

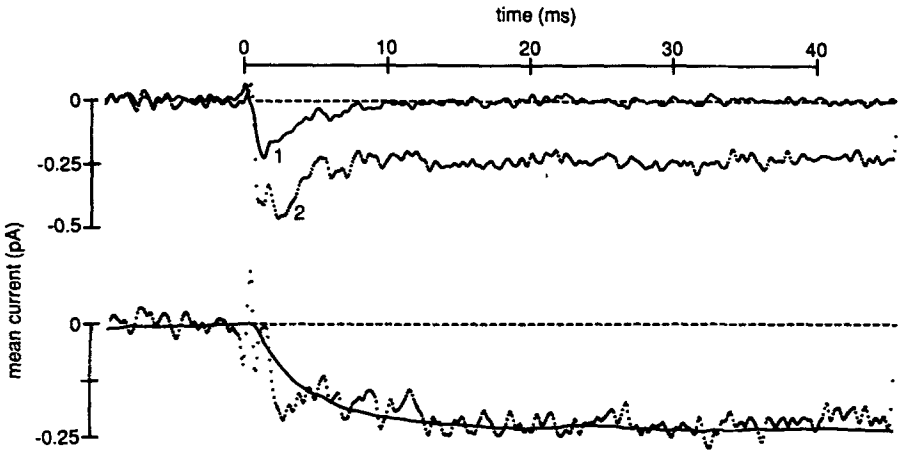


FIGURE 9. Ensemble averages of selected sweeps from the experiment of Fig. 7. The experiment had 468 consecutive sweeps, 23 of which were not used in this figure because they included a modified opening that had started before the sweep began. (*Top*) Mean of all 379 sweeps that did not have any modified events (curve 1). Mean of 66 sweeps with modified openings (curve 2). (*Bottom*) Two ways to estimate the time course of opening of modified channels. The mean current in modified channels was estimated by subtracting 1.67 times curve 1 from curve 2 (dots). The predicted opening time was obtained by scaling the time integral of curve 1 to fit the late part of the mean current (solid line). 250 μM veratridine in the pipette. T = 19°C; f_h = 310 Hz.

approximately. Thus, modified openings start in close temporal association with normal openings.

The second trace in Fig. 7 *B* includes the termination of an unusually short modified event. Not only the beginning but also the termination has a full-sized normal opening, which occurs 32 ms into the depolarization. Such a late normal opening is unlikely to be coincidental. Of the 275 sweeps with only normal openings in this experiment, just two had normal openings occurring as late as this one. We suggest that this pattern represents a conversion of O to VO by binding of veratridine early in the pulse, followed by a reverse transition from

VO back through the normal conducting O state when veratridine dissociates from the channel some 30 ms later. Three other sweeps in this experiment included terminations during the 45 ms of high-frequency sampling that we used, but no normal opening was judged to be associated with the closure of modified channels in these three sweeps.

The association of normal events with openings of modified channels is probably not perfect. One or more modified channels opened during 74 of the 468 depolarizing sweeps. No normal opening was detected in four of them, but this seemed explainable as a detection error. Of the remaining 70 sweeps, 41 had modified openings after a normal opening with no detected delay (<0.5 ms) as was expected; however, 15 had an apparent gap of 1–11 ms between the normal and modified opening (see the fourth trace in Fig. 7 *B*), and 6 had modified openings that began 80–180 ms after the start of the depolarizing step, a time when our lower frequency of sampling did not permit looking for a normal opening with a close temporal association. (No modified openings started in the remaining part of the depolarization.) Finally, eight more sweeps had both normal and modified openings beginning in the first 10 ms of the depolarization, but because they also had another superimposed modified event continuing from the prepulse period, the record was too noisy to analyze for possible short gaps between normal and modified openings. In all, the 74 sweeps above gave rise to 81 modified openings starting during the depolarization. In addition, 18 openings started during the hyperpolarizing prepulse. Again, these openings occurred when we were not sampling at high frequency, but the likelihood that they were associated with an immediately preceding normal opening seems low. These 18 modified openings, which did not appear in Table I or in any of the calculations above, could represent transitions from closed, modified states such as from VI to the open VO state.

DISCUSSION

Comparison with Previous Results

Apparently the actions of veratridine on neuroblastoma cell macroscopic Na currents differ only in detail from those reported earlier for frog nerve and muscle (Ulbricht, 1969*a*; Naumov et al., 1979; Sutro, 1986; Leibowitz et al., 1986, 1987; Rando, 1987). We see the following similarities: Repetitive depolarization reduces the size of the early transient Na conductance while increasing a persistent component. The persistent conductance generated is smaller than the peak transient conductance lost. The rate of induction of the persistent conductance is proportional to the size of the transient Na conductance evoked. The persistent component decays away within seconds after pulsing is stopped and the transient component reappears. At membrane potentials positive to -110 mV, the rate of decay of the persistent component is slightly voltage dependent, slowing with depolarization.

At the single-channel level, we find that veratridine-treated patches have many apparently normal Na channel openings and that modified openings induced by

depolarizing steps are highly correlated in time and number with normal openings. Modified openings are infrequent if the membrane potential is held constant. The slope conductance of modified channels is only 20–25% of that for normal openings, and the mean burst time is ~1 s. Submillisecond closures are frequent throughout the burst, and there are also occasional longer visits to low-conductance substates. These facts suffice to explain the macroscopic observations, including the induction of a small, noninactivating current at the expense of a large peak current during repetitive pulsing and also the long decay time course of the tails.

Our value for single-channel conductance for normal channels agrees in a general way with those given by others in on-cell work with N1E115 neuroblastoma cells (Quandt and Narahashi, 1982; Nagy et al., 1983; Yamamoto et al., 1984; Chinn and Narahashi, 1986) when we take into account the lower temperature and Na^+ concentration and the higher Ca^{2+} concentration used in their work. Our value of the single-channel open time also agrees well with that of Aldrich et al. (1983) when a Q_{10} of 3 is used to correct for a small temperature difference. Many of the single-channel details we report with veratridine are new, but they concur with general observations in bilayer reconstitutions and in an mRNA expression system that veratridine reduces the conductance and prolongs the open time of single Na channels (Levinson et al., 1986; Garber and Miller, 1987; Sigel, 1987). Two abstracts on neuroblastoma cells say that veratridine has no effect on channel conductance but prolongs the open time to 20 ms (Yoshii et al., 1983) or that it reduces conductance by 50% and prolongs the open time to 1.2 s (Yoshii and Narahashi, 1984). We are in closer agreement with the second choice. Our finding that openings of veratridine-modified channels are actually noisy bursts with occasional substates is similar to a description by Chinn and Narahashi (1986) of deltamethrin-modified channels in neuroblastoma cells. With deltamethrin, however, the principal modified channel conductance seems to be the same as for a normal Na channel and the bursting and substates may be less frequent. As the strongest filtering (250 Hz) used in presenting the burst of Fig. 5 is equivalent to the maximum frequency response achieved in experiments with planar lipid bilayers, some of these details would probably not be resolved in many studies using that technique.

The Origin of Modified Channels

The main objective of this article is to identify the pathway(s) from a normal channel to a modified one. There have been two extreme views, as well as a moderate one. One extreme is that at the high veratridine concentrations used here, the drug would react with most Na channels, and that the complex macroscopic currents seen during a depolarization reflect slow opening and closing kinetics of such channels that had already been modified at rest. This view is in part that advanced in the pioneering studies of Ulbricht (1969*a, b,* 1972*a, b*) and is implicit in many studies. The other extreme is Scheme I, in which almost no channels are modified at rest and the only way to modify a normal channel is to open it first. Here the macroscopic "on" kinetics are determined by the kinetics of opening normal channels (Sutro, 1986; Leibowitz et al., 1986). The

middle ground would be that veratridine reacts with both open and closed channels (Scruggs and Narahashi, 1982) or that even if veratridine has not formed the final complex with resting channels, it is at least somehow "preassociated" with them.

This laboratory has shown in frog muscle (Sutro, 1986; Leibowitz et al., 1986, 1987) and now in neuroblastoma cells that during repetitive depolarization with veratrum alkaloids, a transient Na current becomes smaller and a maintained one becomes larger. We have assumed that the transient current represents Na channels that are not modified at rest despite the presence of a high concentration of veratridine. The present work shows that, at the macroscopic level, these currents have the same steady state inactivation and peak activation properties that are found in untreated cells; at the single-channel level, they come from openings with conductances and open times like those in untreated cells. Thus, there is no evidence that these brief openings represent anything but unmodified Na channels.

Our working hypothesis has been that unmodified Na channels become modified by veratridine when they are open. This was suggested by the finding that an induction of tail current occurred whenever a depolarization elicited a normal current and that the rate of induction was proportional to the integral of the preceding normal Na current (Sutro, 1986). The tail ceased growing if normal Na channels became inactivated, and the growth could be prolonged if inactivation were slowed or removed by scorpion toxins, chloramine-T, or *N*-bromoacetamide. Now we have pursued this hypothesis at the single-channel level. Regrettably, we did not obtain patches with only one Na channel, where the fate of the channel could be followed without ambiguity. However, in the multichannel experiment that we analyzed most carefully, we found within experimental error that every modified opening appearing soon after a depolarizing step was preceded by one or more normal openings and that the latency curve for modified openings could be predicted from the time course of opening of normal Na channels. We could rule out the hypothesis that the similarity in opening times arises merely because pre-existing modified channels happen to have gating kinetics that resemble those of normal channels. Instead, we could show that modified channels appear in a subset of sweeps that is significantly enriched in normal channel openings. These observations agree with the hypothesis that modified channels come from open ones. Taking into account the error of missed openings, we find under our conditions that the probability that an open, normal channel becomes modified is 0.10 per opening. Factoring in the 253-μs mean open time of these channels gives an effective rate constant for the O-to-VO transition of 400 s^{-1} (250 μM veratridine) and a bimolecular rate constant of 1.6×10^6 $M^{-1}s^{-1}$. The two other patches analyzed in the same way gave bimolecular rate constants of 4.5 and 2.0×10^6 $M^{-1}s^{-1}$. These numbers confirm an earlier estimate of 2×10^6 $M^{-1}s^{-1}$ made from macroscopic currents using several additional assumptions (Leibowitz et al., 1986).

Are these results also consistent with alternative hypotheses? Consider the extreme case where veratridine reacts only with closed channels, so that all modified openings during a depolarization come from modification events that

occurred before the depolarizations. This hypothesis predicts that there should be a negative correlation between the number of normal openings and the number of modified openings in each sweep since, when a channel becomes modified at rest, it will not be available to give a normal opening during the depolarization. The prediction is contradicted by the statistical results of Table I and Fig. 8 with three carefully analyzed patches. Each patch gives a strong positive correlation. Consider the more relaxed case where modification occurs with equal likelihood and rates to open or closed channels. This hypothesis predicts no use dependence and, for the same reason as before, a negative correlation. It, too, is contradicted by the observations.

The details of the modification event would be much easier to confirm in one-channel patches. In <25% of our records, a gap of a few milliseconds appears to separate the normal opening from the modified opening. This raises the possibility that the process of modification may sometimes or always close the channel transiently. The short closure would reflect conformational adjustments needed to go from the high-conductance, quickly inactivating normal state to the lower-conductance, nearly noninactivating modified state. Alternatively, instead of being fully closed, the channel might be passing through a poorly conducting substate like that seen in the strongly filtered records of Fig. 5. We have also seen but not emphasized some bursts of modified openings with a conductance different from the most common one. These might reflect molecular heterogeneity of Na channels in N18 neuroblastoma cells. Nagy et al. (1983) have reported that normal Na channels exhibit more than one conductance level in neuroblastoma cells as well.

In this article, we have emphasized the creation of modified channels from normal ones and ignored two other pathways to the modified open state in Scheme I, namely those from the modified, closed states designated VI and VO. Such transitions might account for the occasional late openings (after many seconds) seen during long depolarizations as in Fig. 6 *A*, the small class of openings during the hyperpolarizing pulse, and the smaller class of openings between 80 and 180 ms after a depolarizing pulse. In general, all of these classes of openings seem to become more frequent and longer lasting after a train of depolarizations. When we simply hold the membrane potential steady at one voltage, "spontaneous" modified openings become less and less frequent over a time course of tens of seconds. We have also not considered in detail the eventual return from the modified to the normal states (see Rando, 1987).

Because of the considerable evidence that veratridine binds to the same receptor as aconitine, batrachotoxin, and grayanotoxin (Catterall, 1977; Hille et al., 1987), we imagine that the forward reaction with all of these toxins shares in a general way the features that we have described here for veratridine. It has been to our advantage that the reaction with veratridine is so short lived, allowing us to repeat the modification event again and again. Since the reactions with batrachotoxin and aconitine are nearly irreversible (Schmidt and Schmitt, 1974; Khodorov, 1985), the chance of observing the modification event with them would be vanishingly small. The electrophysiological literature confirms that modification with all of these toxins is greatly speeded by repeated opening of

Na channels (Mozhayeva et al., 1977; Khodorov and Revenko, 1979). Nevertheless, they act in situations where repetitive depolarizations and repolarizations are not applied: veratridine will make a significant standing current when applied to a steadily depolarized node of Ranvier (Ulbricht, 1969*b*, 1972*a*), and batrachotoxin and veratridine can be used to initiate Na fluxes in cell cultures and to open Na channels incorporated in lipid vesicles or planar bilayers (Catterall, 1977, 1986; Krueger et al., 1983; Garber and Miller, 1987). It remains an interesting and unanswered question how the modified channels arise in these cases. One would like to design experiments to ask whether spontaneous normal openings explain the observed rate of modification or whether other pathways (C $\rightharpoonup$ VC, I $\rightharpoonup$ VI) play a major role.

Note added in proof: After submission of this article, Sigel (1987, *Pflügers Archiv*, 410:112–120) further described veratridine modification of Na channels from chick mRNA expressed in *Xenopus* oocytes. The modified single-channel events had a burst time and relative conductance similar to ours.

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