

Rapid and Slow Gating of Veratridine-modified Sodium Channels in Frog Myelinated Nerve

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ABSTRACT The properties of voltage-dependent Na channels modified by veratridine (VTD) were studied in voltage-clamped nodes of Ranvier of the frog *Rana pipiens*. Two modes of gating of VTD-modified channels are described. The first, occurring on a time scale of milliseconds, is shown to be the transition of channels between a modified resting state and a modified open state. There are important qualitative and quantitative differences of this gating process in nerve compared with that in muscle (Leibowitz et al., 1986). A second gating process occurring on a time scale of seconds, was originally described as a modified activation process (Ulbricht, 1969). This process is further analyzed here, and a model is presented in which the slow process represents the gating of VTD-modified channels between open and inactivated states. An expanded model is a step in the direction of unifying the known rapid and slow physiologic processes of Na channels modified by VTD and related alkaloid neurotoxins.

INTRODUCTION

The alkaloid neurotoxin veratridine (VTD) exerts its toxic effects by modifying voltage-dependent Na channels in nerve and muscle (Ulbricht, 1969). VTD may modify Na channels by binding rapidly to open channels (Sutro, 1986; Rando, 1987*b*). There is also a VTD-modified permeability that develops over several seconds under the condition of prolonged membrane depolarization (Ulbricht, 1969). By whichever pathway the modification is effected, the modified current persists when the membrane is depolarized and declines over several seconds when the membrane is held at -100 mV.

The VTD-modified channel gates rapidly between an open and a closed state (Leibowitz et al., 1986; Rando, 1987*b*). Leibowitz et al. (1986) demonstrated that in frog muscle, the kinetics of that gating reaction could be described by the sum of two exponential processes, suggesting that there are two closed states, but that the energy of activation of the channel was the same as that for unmodified channels. Ulbricht (1969) described the slowly developing VTD-modified permeability in frog nerve as the gating of channels between a modified closed state and a modified

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open state. This transition had exponential kinetics, although a thousand times slower than unmodified channels, and a much greater energy of activation than unmodified channels.

The purpose of this investigation was to study both rapid and slow transitions of the VTD-modified Na channel in the same tissue, the frog node of Ranvier. Studies of the rapid gating process revealed important qualitative and quantitative differences in nerve compared with muscle. Investigation of the slow transitions of the VTD-modified channels and the dissociation of VTD from the channel led to a reinterpretation of the slowly developing and slowly declining VTD-modified permeability. A kinetic scheme is presented to unify the rapid and slow transitions of the VTD-modified channels. Some of these results have been presented in preliminary form (Rando, 1987a).

METHODS

Single myelinated fibers were isolated from sciatic nerves of the frog *Rana pipiens*. Fibers ranged in diameter from 10 to 15 μm and were voltage-clamped according to the method of Dodge and Frankenhaeuser (1958).

The fibers were dissected in a standard Ringer's solution containing 110 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl_2 , 12.0 mM tetraethylammonium chloride (to block the delayed K current), and 5.0 mM HEPES buffer adjusted to pH 7.2 with 1 N NaOH. The fibers were mounted in a plexiglass chamber and petroleum jelly (Vaseline) seals were laid down beneath the surface of the solution. The solution level was lowered creating the four pools described by Dodge and Frankenhaeuser (1958). The solution in the end pools was then changed to an "intracellular" solution of 110 mM CsCl, 10 mM NaCl, and 5.0 mM HEPES, adjusted to pH 7.2 with 1 N CsOH. The adjacent internodes were cut again allowing the intracellular solution to diffuse to the inside of the node. The plexiglass chamber was transferred to the voltage-clamp apparatus where cooled agar bridges (stored in 1 M KCl) were connected to each pool. A cooling solution that was circulated through the base plate of the apparatus maintained the preparation at $12 \pm 2^\circ\text{C}$.

The holding potential was set at -100 mV. Linear leakage and capacitance currents were subtracted using analogue circuitry and current traces were filtered with an active four-pole Bessel low-pass filter (3200; Krohn-Hite Corp., Avon, MA) at 10 kHz when measuring responses on a millisecond time scale, and 1 kHz for responses on a time scale of seconds. All data were displayed on an analogue storage oscilloscope (5441; Tektronix Inc., Beaverton, OR), photographed with an oscilloscope camera (Tektronix C-5B), and digitized for analysis using a Digiplot (Houston Instrument Co., Austin, TX) in conjunction with an eight-bit microcomputer (Horizon 2; North Star Computers, San Leandro, CA). Some of the figures are hand tracings of the photographic records for clarity as indicated. The currents were calibrated by dividing the measured internodal potential differences by an assumed internodal resistance of 20 $\text{M}\Omega$. Where indicated, Na currents have been converted to Na permeabilities using the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin and Katz, 1949). Wherever data represent the mean of several experimental results, the error values or error bars indicate $\pm\text{SD}$ about the mean.

VTD was obtained from Sigma Chemical Co., St. Louis, MO. Solutions containing VTD were prepared by diluting the drug from a 20-mM stock solution in dimethylsulfoxide (DMSO). The highest concentration of DMSO in any solution in these experiments (1.58%) had no effect on nodal Na currents. All experiments were done in the continued presence of 200 μM VTD unless otherwise indicated.

"Instantaneous" currents are described that refer to the current measured upon changing the clamp potential from one value to another. The settling time of the voltage clamp is $\sim 30 \mu\text{s}$ and thus would contribute an error of $<5\%$ toward the estimation of the true instantaneous current at time zero over most of the potential range studied (-180 to -80 mV). For the extreme potentials of <-180 mV and >-80 mV, where the error could approach 10%, extrapolation of the currents (which were all changing exponentially) back to time zero eliminated the error introduced by the clamp settling time.

RESULTS

The modulations of VTD-modified Na permeability in frog nerve are complex functions of voltage and time. Apparent steady state permeabilities can be attained within several milliseconds in response to changes in membrane potential. However, these rapidly attained "steady state" currents, at constant membrane potential, slowly change over several seconds reaching a new steady state level. The time courses of these processes differ by three to four orders of magnitude and thus can be studied essentially independently.

The results described below are divided into two sections. The first concerns the rapid gating of VTD-modified Na channels, with time constants in the order of 1 ms or less. The second section concerns a slower gating reaction which is measured over several seconds.

The Rapid Gating of the VTD-modified Permeability

When VTD was applied to a voltage-clamped myelinated axon, the family of Na currents elicited by positive voltage steps appeared to be little affected (Fig. 1). The magnitude and time course of the transient currents seemed virtually unchanged compared with those in the control Ringer's solution. When the membrane was returned to the holding potential, an inward current persisted, indicated by the arrow in Fig. 1 B. The effect of VTD is shown more clearly in Fig. 1 C, a tracing from another axon in which VTD produced more dramatic changes. In the absence of VTD, a single pulse to $+100$ mV produced a transient outward current that inactivated within 2 ms. In the presence of VTD, the same pulse produced a transient current of the same amplitude, but the current did not inactivate completely. Rather, there remained a persistent outward current at that potential. When the membrane was repolarized to -100 mV, this current became an inward current that decayed to zero over several seconds. This persistent current flows through only VTD-modified channels, thus the gating characteristics of such channels can be studied independently of unmodified channels.

VTD-modified currents generated by brief depolarizing pulses followed a characteristic pattern when the membrane was repolarized to -100 mV. There was a rapid, small decay of the current that was complete within several milliseconds, after which the current maintained an apparent steady state level (Fig. 2). If the membrane were instead repolarized to -200 mV, the initial decay of the current was greater and more rapid, and the steady state current was smaller. Upon the return of the membrane to -100 from -200 mV, the current increased and reached the same level as that when the membrane was repolarized to -100 mV initially.

The greater reduction of the current when the membrane was repolarized

to -200 mV was not due to dissociation of VTD from Na channels. If it were, then there would have to be a reassociation process to explain the increase of the current upon a return to -100 from -200 mV. However, there is evidence that no association of VTD with channels occurs with a step from -200 to -100 mV; that is, in the absence of a depolarizing prepulse, hyperpolarization of the membrane to -200 mV followed by a step to -100 mV resulted in no VTD-modified current. Thus any dissociation of VTD from channels at -200 mV would have manifested itself as a

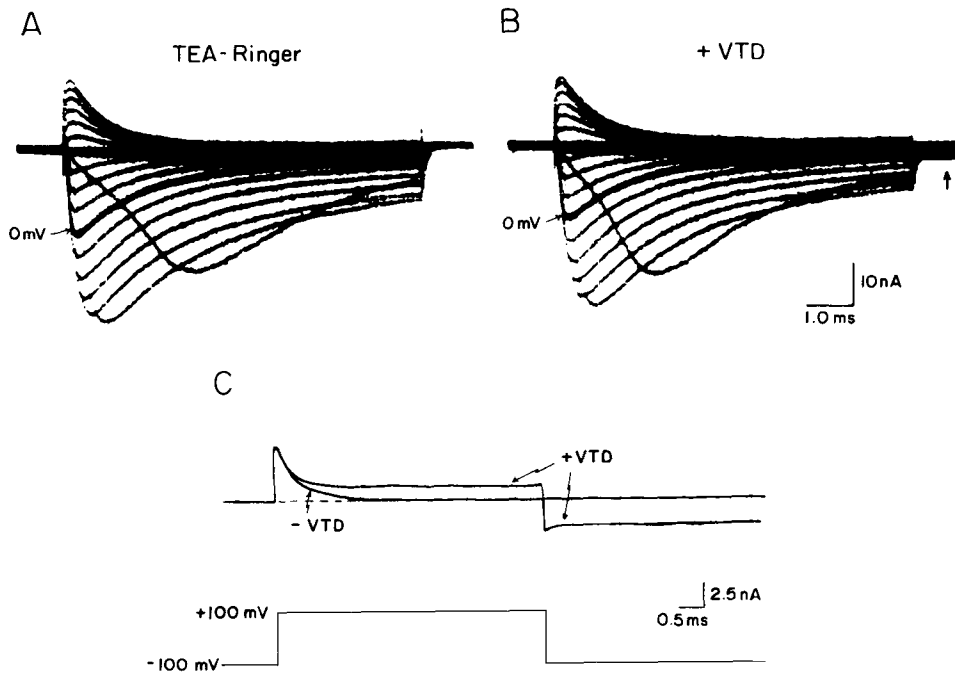


FIGURE 1. Na currents in the node of Ranvier in the absence and presence of VTD. With the nodal membrane under voltage-clamp control, currents were generated by 8-ms depolarizations in 10-mV increments from a holding potential of -100 mV. (A) A family of Na currents in standard Ringer's solution. (B) A family of Na currents 5 min after the addition of $200 \mu\text{M}$ VTD to the Ringer's solution. Note the small persistent current following the test depolarizations (arrow). (C) Na currents from a different node in the presence and absence of $200 \mu\text{M}$ VTD. A single 5-ms pulse to $+100$ mV was delivered before and 1 min after the addition of VTD.

smaller modified current upon return to -100 mV. VTD remained associated with the channels at -200 mV, yet the modified channels were in a nonconducting state. Since these modified channels were readily opened with a depolarization to -100 mV, this process represents the conversion of channels from resting to open states.

I studied the voltage dependence of this gating process by inducing VTD-modified currents and then stepping the membrane to new test potentials. For test potentials more positive than -60 mV, the current at the end of the test pulse var-

ied nearly linearly with membrane potential (Fig. 3, A and C). For test potentials more negative than -60 mV, the current at the end of the test pulse did not vary linearly but decreased as the test potential was made more negative (Fig. 3, B and C).

The currents at the end of the test pulse were not truly at steady state but were slowly changing (see below): they were decreasing for pulses more negative than ~ -80 mV (the slowly decaying current) and they were increasing for pulses more positive than ~ -80 mV (the slowly developing VTD-modified current). Thus, I refer to the relationship of the current at the end of the test pulse (10 ms) to the test pulse potential as an isochronal current-voltage (I - V) relationship. This is to be distinguished from the steady state I - V relationship of VTD-modified channels (Fig. 7 B).

Although the shape of the isochronal I - V curve (Fig. 3 C) resembles that of unmodified channels, the two curves differ in several ways. First, the reversal poten-

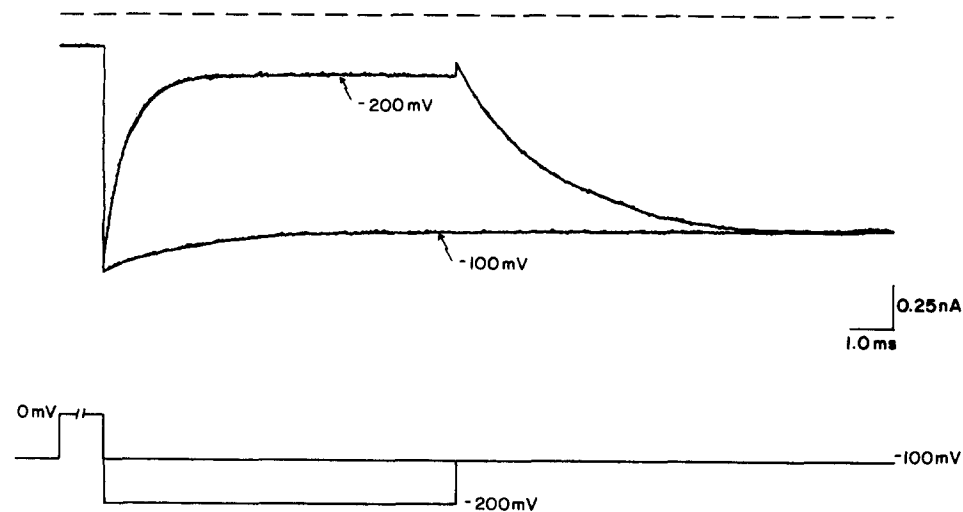


FIGURE 2. VTD-modified channels gate between resting and open states. A VTD-modified current was induced by a 20-ms depolarization to 0 mV. The nodal membrane was then hyperpolarized to -100 or -200 mV and tracings of the resulting currents are superimposed here. The dashed horizontal line in this and all subsequent figures of current traces represents the zero-current level. After 8 ms at -200 mV, the membrane was depolarized back to -100 mV. The modified current increased to the same level as when the membrane had been repolarized to -100 mV initially.

tial for the modified channels was less positive than that of unmodified channels (modified, 23.7 ± 3.4 mV; unmodified, 46.6 ± 3.7 mV; $n = 6$). This shift in reversal potential is consistent with a decrease of Na selectivity of channels when modified by VTD (Rando, 1987b). Second, the modified channels conducted a maximum inward current at test potentials of -80 mV, compared with -30 mV for unmodified channels. This was not simply a function of the shift of the reversal potential since the permeability-voltage relationship was also shifted to more negative potentials (see below). Finally, current continued to flow through VTD-modified channels even at

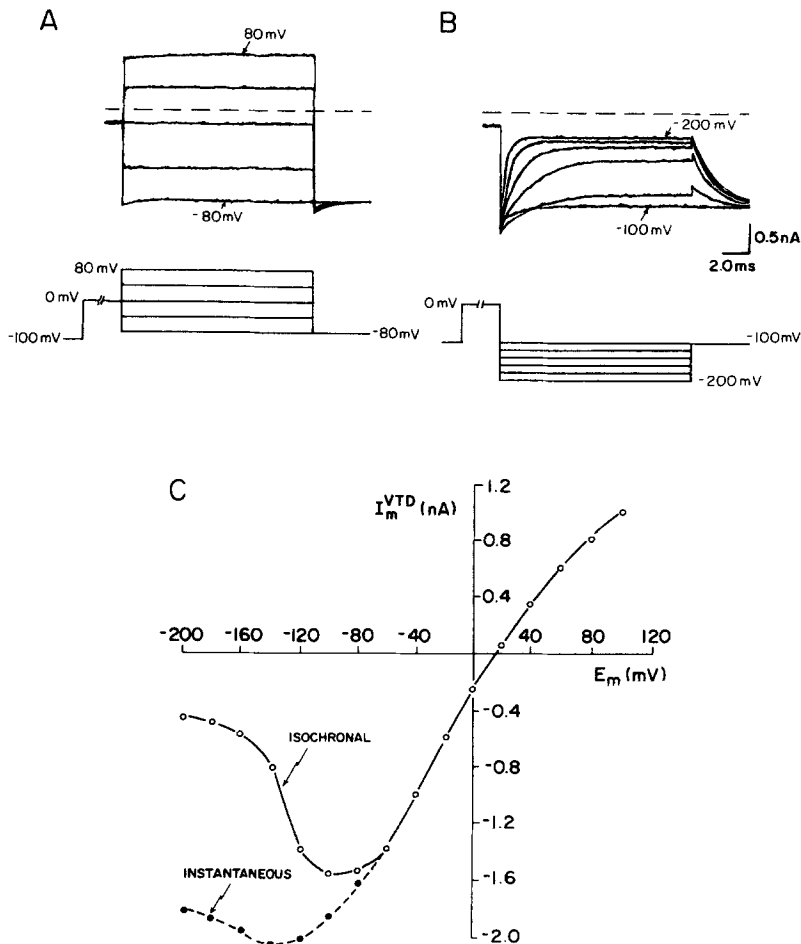


FIGURE 3. I - V relationships for VTD-modified channels. VTD-modified currents were induced with 20-ms depolarizations to 0 mV, and the membrane was then stepped to test potentials for 10 ms. (A) Test pulse range, -80 to $+80$ mV. (B) Test pulse range, -200 to -100 mV. At each new potential, the current instantaneously changed and then, for test potentials more negative than -60 mV, decreased to reach an apparent steady state level (see text). The current at the beginning of the test pulse is referred to as the instantaneous current and that at the end of the test pulse (10 ms) as the isochronal current. (C) The values of the instantaneous and isochronal currents for VTD-modified channels are plotted as a function of the test potential.

potentials as negative as -200 mV, whereas current through unmodified channels decays completely with hyperpolarization of the membrane beyond ~ -60 mV.

Instantaneous Rectification of VTD-modified Channels

VTD-modified currents were reduced at hyperpolarized potentials for two reasons. First, as just described, there was a voltage-dependent gating reaction that favored a closed state as the membrane was increasingly hyperpolarized. Second, there was a

voltage-dependent reduction of the current through the open channels. If the conductance of the open channels were independent of voltage, then the instantaneous current would be a linear function of membrane potential. However, the instantaneous current did not increase linearly, but rectified with membrane potentials more negative than -60 mV (Fig. 3 C). This rectification is consistent with voltage-dependent Ca block of the channels, as has been described for both batrachotoxin-modified and tetramethrin-modified Na channels (Mozhayeva et al., 1982; Yamamoto et al., 1984). Thus, both voltage-dependent gating and open channel rectification contributed to the reduction of the VTD-modified current at membrane potentials more negative than the normal resting potential.

Permeability-Voltage Relationship of VTD-modified Channels

The isochronal I - V relationship of VTD-modified channels was converted to a permeability-voltage relationship in Fig. 4 A (solid line). The dashed line shows the same data corrected for open channel rectification.¹ The modified channels showed voltage-dependent gating over the range of -180 to -60 mV. As mentioned above, a fraction of the modified channels did not close, even at -200 mV. This current was not an artifact of improper leakage subtraction since hyperpolarization of the membrane to -200 mV without a previous depolarization resulted in no inward current. Furthermore, this current was sensitive to block by tetrodotoxin and thus was mediated by voltage-dependent Na channels.

The permeability-voltage relationship of the VTD-modified channels and of unmodified channels is shown in Fig. 4 B. The curve representing the VTD-modified channels is normalized to exclude the fraction of channels that remained open at -200 mV. The solid line through the points for the activation of unmodified channels is drawn according to the equation:

$$A = \frac{1}{1 + \exp [(E_o - E_m)/k]}, \quad (1)$$

where A is the ordinate value of fractional activation, E_m is the abscissa value of membrane potential, E_o is the membrane potential at which $A = 50\%$, and k is a slope factor. For the unmodified channels, E_o was -30 mV and k was 5.16 mV. The solid line through the points for the activation of VTD-modified channels was drawn according to the model presented in the Discussion, but it is fit by Eq. 1 with $E_o = -120$ and $k = 12.0$ mV. The dashed line shows the curve through the points for unmodified channels when shifted by -90 mV (i.e., $E_o = -120$ mV, $k = 5.16$ mV). The curve for the VTD-modified channels is clearly less steep than the curve for unmodified channels. A model for this voltage-dependent gating of VTD-modified channels is presented in the Discussion.

¹ The correction for open channel rectification was done as follows. The permeability of the VTD-modified channels was calculated using the Goldman-Hodgkin-Katz equation for the linear portion of the I - V curve (between -60 and 0 mV). Then the current expected at more negative potentials for that permeability was calculated using the same equation. The ratio between the calculated and the observed values of the instantaneous current, as a function of membrane potential, was the correction factor in the permeability-voltage relationship in Fig. 4 A.

Kinetics of Opening and Closing of VTD-modified Channels

There was a time-dependent closing and reopening of VTD-modified channels when the membrane potential was changed to a new value (Figs. 2 and 3). At every membrane potential tested, both the opening and closing processes were well fit by single exponential functions. These relaxations proceeded without any apparent delay after each voltage step. The time constants of these relaxations as a function of membrane potential (Fig. 5) were determined by fitting both the closing process (closed circles) and the reopening process (open circles) with exponential functions.

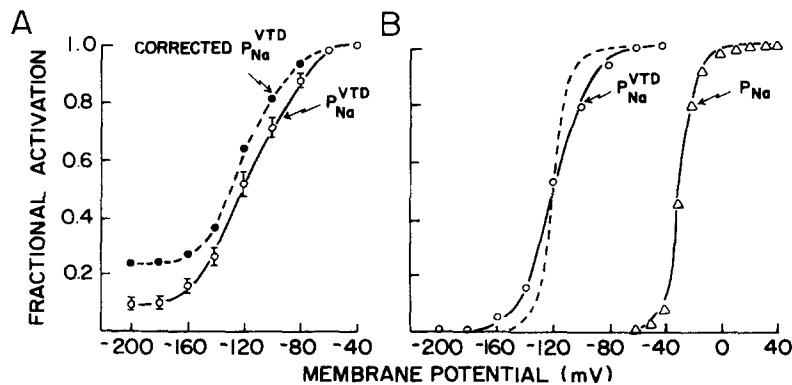


FIGURE 4. Permeability-voltage relationships for VTD-modified channels. (A) Isochronal current values (obtained as in Fig. 3) were converted to permeabilities and plotted as a function of membrane potential (P_{Na}^{VTD}). Each symbol represents the mean value from six nodes and, where error bars are not shown, the standard deviation was smaller than the size of the symbol. The mean permeability at each potential was then corrected (corrected P_{Na}^{VTD}) for open channel rectification as seen in the instantaneous I - V relationship of Fig. 3 (see text for discussion of the correction factor). This corrected curve should represent the voltage-dependent gating of VTD-modified channels over this potential range. (B) The corrected P_{Na}^{VTD} curve of A was normalized from its minimum to its maximum value on a scale of 0–1.0 and is shown as the open circles (P_{Na}^{VTD}). Also shown is the voltage-dependence of the activation of unmodified channels (triangles, P_{Na}). The solid lines through the triangles are drawn according to Eq. 1 and the values given in the text; the solid lines through the circles are drawn according to the model presented in the Discussion. The dashed line is the solid curve through the triangles shifted -90 mV along the abscissa.

The solid line in Fig. 5 represents the time constants predicted from the model for these transitions as presented in the Discussion.

Because the gating of VTD-modified channels in frog muscle has been described as having two components, a rapid component analogous to that described here and a slower component (Leibowitz et al., 1986), I analyzed the records carefully for a slow component in nerve. No slow component was found. In particular, I considered the possibility that the fraction of the current that persisted at -200 mV was a slowly decaying current. However, no change of this current was detectable over the final 12 ms of 15-ms test pulses. If this current had been slowly changing, but to an extent equal to the error in the measurements so that it was not detected, then the

time constant of that decay would have to be >100 ms at -200 mV; the maximum slow time constant observed in muscle was <20 ms (Leibowitz et al., 1986).

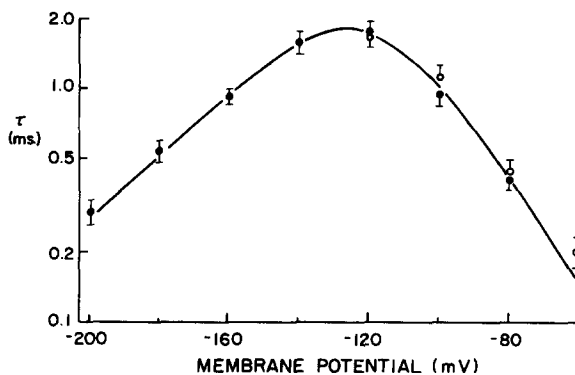
Slow Changes of the VTD-modified Permeability

The VTD-modified permeability induced by a brief depolarization, as in Fig. 1, was not at steady state but decayed over several seconds when the membrane was repolarized. The decay was exponential and, in nine fibers treated with $200 \mu\text{M}$ VTD, the time constants of the decay at -100 mV averaged 2.21 ± 0.21 s. The rate of decay was independent of VTD concentration over a range of 10 to $316 \mu\text{M}$ (Rando, 1987b). The nature of this slowly decaying current is examined below.

Voltage Dependence of the Decay of the VTD-modified Current

To test if the decay rate of the modified current was dependent on the membrane potential, the repolarization potential was varied (Fig. 6). The decay of the modified

FIGURE 5. Time constants of relaxation of VTD-modified channels. The relaxation of VTD-modified currents as shown in Figs. 2 and 3 could be well fit by single exponential functions. The time constants of those processes are shown as a function of pulse potential. The solid circles represent the time constants calculated from the relaxation that occurred as the membrane was stepped from 0 mV to the indicated potentials; the open circles represent the relaxation time constants as the membrane was stepped from -180 or -200 mV to the indicated potentials. Each point represents the mean of determinations from six different nodes. The solid line is drawn according to the model presented in the Discussion.



current was exponential for potentials between -80 and -140 mV, and within that range the decay rate increased with increasingly negative repolarization potentials. I was unable to clamp the membrane to potentials more negative than -140 mV for a time sufficient to measure a time constant without doing irreversible damage to the nodal membrane. An increase in the decay rate of the VTD-modified current at increasingly negative potentials was also observed in a preliminary study by Yoshii and Narahashi (1984).

The Slowly Developing VTD-modified Permeability

As mentioned above, a slowly developing VTD-modified current appears during depolarizing pulses that last many seconds; this was originally described by Ulbricht (1969). This slowly developing permeability may represent the slow binding of VTD to channels or the slow opening of channels already modified by VTD. From the

results of the following experiments, it seems as if the latter of these two possibilities more accurately describes the process.

Time and Voltage Dependence of the Slow VTD-modified Current

When a node was depolarized to various potentials for many seconds in the presence of VTD, an inward current increased slowly in an exponential fashion (Fig.

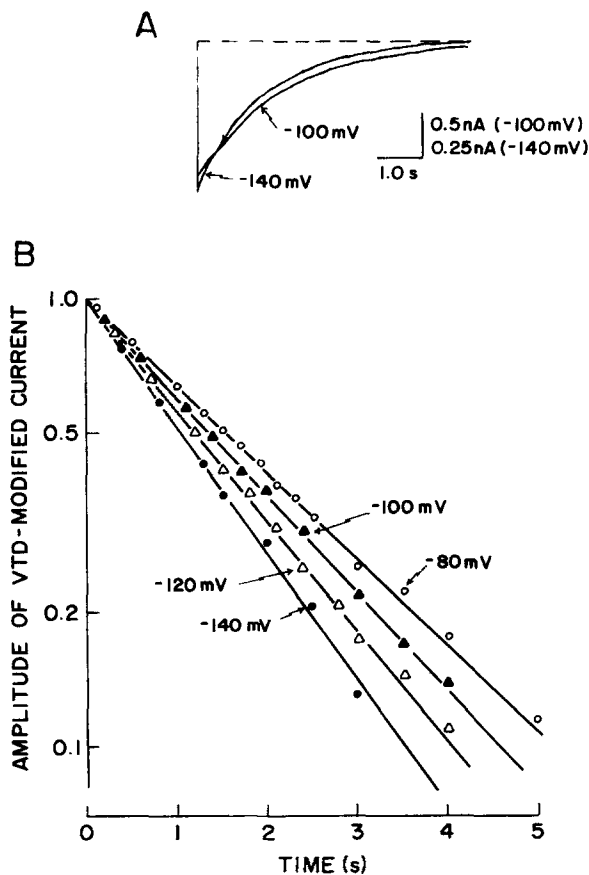


FIGURE 6. Voltage dependence of the rate of decay of the VTD-modified current. VTD-modified currents, generated with 10-ms pulses to 0 mV, were found to decay exponentially with different time constants at different repolarization potentials. (A) Superimposed tracings of the decay of the VTD-modified current at -100 and -140 mV. The trace at -140 mV has been scaled by a factor of two for easier comparison. The initial amplitude of the current at -140 mV is smaller than that at -100 mV because of instantaneous rectification and voltage-dependent closing of VTD-modified channels at negative membrane potentials (Fig. 3 C). (B) The time course of decay of the VTD-modified current at the indicated membrane potentials are normalized and plotted semilogarithmically. The currents decay exponentially at all potentials but the time constants decrease with hyperpolarization: $\tau = 2.20$ s (-80 mV), 1.95 s (-100 mV), 1.73 s (-120 mV), and 1.51 s (-140 mV). Averaged values of these time constants from several experiments are presented in Fig. 10.

7 A). This slow current reached a steady state within 8 s at all pulse potentials. After each depolarization, there was an exponentially decaying current whose time constant was in the same range as those generated by brief depolarizations (Fig. 6). The steady state I - V and permeability-voltage relationships are shown in Fig. 7, B and C.

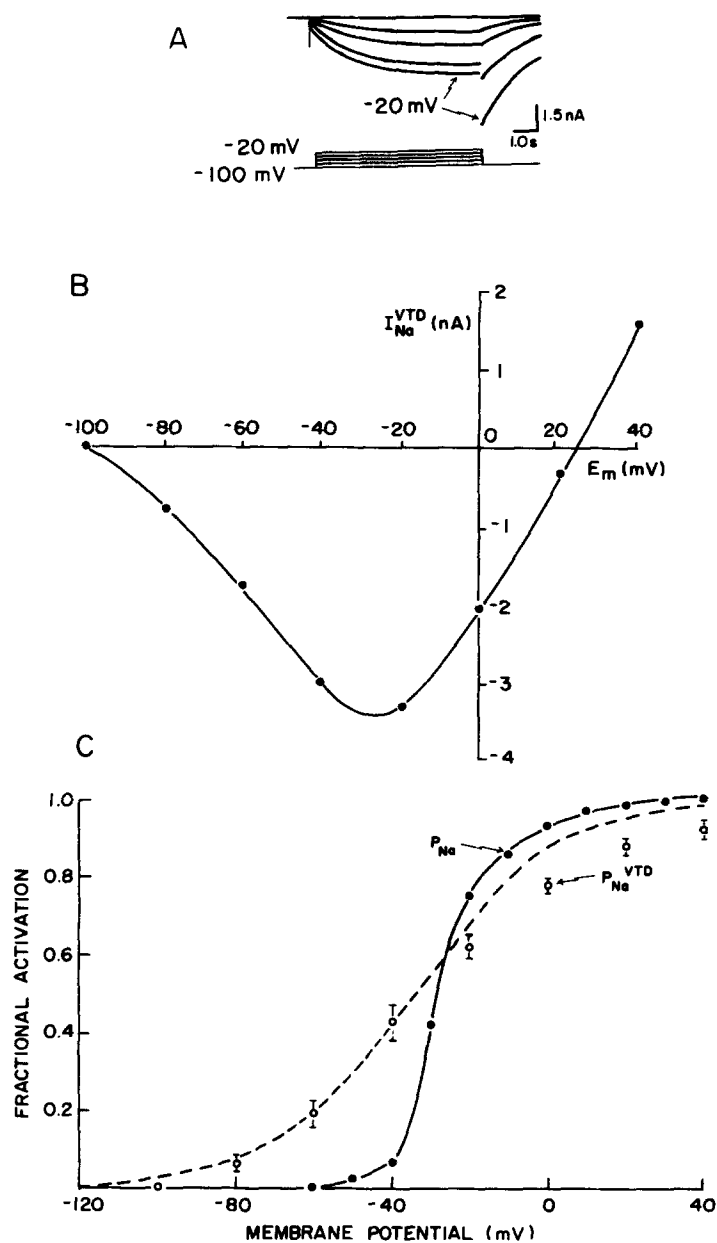


FIGURE 7. Slowly developing VTD-modified permeability. (A) A node was depolarized for 7 s to various test potentials. These depolarizations produced slowly developing inward currents and, upon repolarization, slowly decaying currents. Superimposed tracings of currents in response to depolarizations to -80, -60, -40, and -20 mV are shown ($[VTD] = 200 \mu M$). (B) Steady state $I-V$ relationship. These steady state current levels are plotted as a function of depolarizing potential. The nodal membranes would not withstand prolonged polarizations to potentials more positive than +40 mV, and often not beyond 0 mV. (C) The values of the steady state currents from six nodes were converted to permeabilities and plotted as a function of pulse potential (open circles). The most positive potential achieved without instability of the voltage clamp was +40 mV in three nodes, +20 mV in one node, and 0 mV in two nodes. The modified permeability was not at a maximum at +40 mV, so the normalization was arbitrarily determined by the model presented in the Discussion. The dashed curve is drawn according to the model. Included in the graph is the permeability-voltage relationship for unmodified channels from the same node used in A and B (closed circles).

In agreement with Ulbricht's report (1969), this slow process was detectable at potentials ~ 30 mV more negative than the minimum potential for detecting the activation of unmodified Na channels. Furthermore, compared with the peak permeability of unmodified channels, the steady state permeability of the VTD-modified channels was a less steep function of voltage.

The reversal potential of the steady state current is considerably less positive than that of the peak Na current (48.7 ± 3.5 mV for the six nodes described below). The average reversal potential of the VTD-modified current from three nodes was 27.6 ± 1.5 mV. From three other nodes that could not be polarized to such positive potentials, estimates of the reversal potentials from extrapolation of the steady state *I-V* curves were all between 25 and 35 mV. This change of reversal potential in the presence of VTD is due to a reduction of the selectivity of the channels for Na over the carriers of outward current, K and Cs (Rando, 1987*b*).

The slow current developed with an exponential time course from the zero-current level for depolarizations to potentials more negative than ~ -60 mV. For potentials more positive than -60 mV, the transient Na current was activated and thus the fast VTD-channel interaction occurred (as in Fig. 1). The slow current then developed exponentially from this small current level. The fast interactions appear as initial jumps in the currents for pulses to -40 and -20 mV in Fig. 7 *A*.

Another way to study the development of the slow permeability is to measure the initial amplitude of the slowly decaying current as a function of pulse duration; as the slow permeability develops, so will the amplitude of this current (Fig. 8 *A*). When the initial amplitudes were plotted semilogarithmically as a function of pulse duration for a depolarization to -20 mV, the exponential nature of the development of the slow permeability was evident (Fig. 8 *B*). Similar analyses for pulses to -40 and -60 mV showed that over this voltage range the development of the slow permeability continued to be a first-order process with a time constant that decreased with increasing depolarization (Fig. 8 *B*). This type of analysis was not done for depolarizations more positive than -20 mV because of the difficulty of maintaining a node polarized at those potentials.

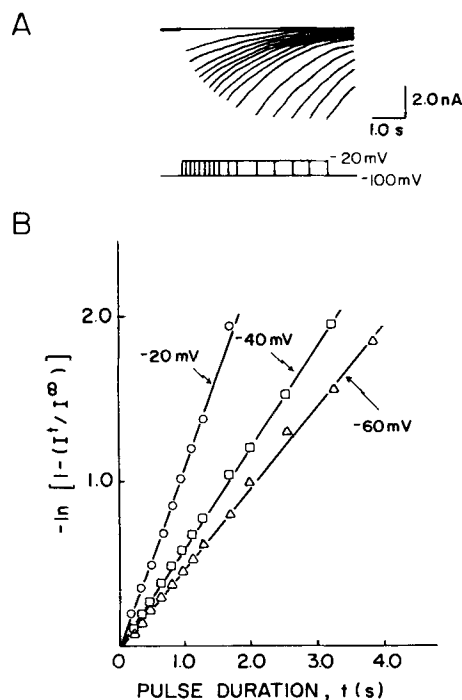
The slowly developing VTD-modified current was studied using different VTD concentrations. The amplitude of the current at steady state increased as the VTD concentration was increased from 60 to 200 μM . The time course of that development, however, as studied in Fig. 8, was independent of VTD concentration over this concentration range. In four nodes, the time constants at -40 mV, derived as in Fig. 8, were 1.62 ± 0.08 s at 60 μM VTD, 1.60 ± 0.07 s at 100 μM , and 1.62 ± 0.10 s at 200 μM VTD. This would suggest that the slowly developing current cannot be equated with the slow binding of VTD.

Although the slow current developed with a very different time course and voltage dependence from the rapid modification of channels by VTD, the resulting permeabilities of the two processes appeared to be identical by three criteria. First, when the membrane was repolarized after a prolonged depolarization, the slowly decaying current had identical kinetics to that after a brief depolarizing pulse. Second, the rapid gating of VTD-modified channels described above was the same whether the modification was induced by a brief or a prolonged depolarization.

Finally, the reduction of selectivity of VTD-modified channels for Na over K was the same regardless of how the modification was achieved (Rando, 1987b).

The relationship between the slowly decaying current after a brief depolarization and the slowly developing current during a prolonged depolarization is clarified by the comparison of Figs. 6 and 8. The first implies a process by which channels convert from a conducting to a nonconducting state with first-order kinetics, in a voltage-dependent manner, and with a time constant of $\sim 1-2$ s. The second implies a process by which channels convert from a nonconducting to a conducting state with

FIGURE 8. Voltage dependence of the rate of development of the VTD-modified permeability. (A) A node was depolarized to -20 mV with prolonged pulses of various durations. The slowly developing inward current, as shown in Fig. 7, is not displayed, but tracings of the decaying currents after the pulses are shown. The envelope of these currents represents the time course of development of the slow VTD-modified current. (B) The data from experiments like that in A are plotted on a semi-logarithmic graph to show the exponential nature of the time course of the slow VTD-modified current. The initial jumps of the currents from the rapid VTD channel interaction (see Fig. 7) were subtracted out. The value of I' represents the amplitude of the modified current after a depolarization of t seconds; the value of I^∞ represents the amplitude of the current at steady state. The different symbols are from experiments with different pulse potentials as indicated. The time constant of the process decreased as the pulse potential was made more positive: $\tau = 2.08$ s (-60 mV), 1.69 s (-40 mV), and 0.92 s (-20 mV). Averaged values of these time constants from several experiments are presented in Fig. 10.



first-order kinetics, in a voltage-dependent manner, and with a time constant of $\sim 1-2$ s. The possibility that these two processes are manifestations of a single gating reaction is considered in the Discussion.

Reversal of the Interaction between VTD and the Na Channel

The VTD-modified current decayed slowly when the membrane was returned to the holding potential. This decay was the same whether the modification was induced by

a brief depolarization as in Fig. 1, or by a prolonged depolarization as in Fig. 7. What is the process responsible for the decline of this current? There are two general possibilities. First, the conducting channels may remain modified by VTD but enter a nonconducting state with the observed time course at -100 mV. Second, VTD may dissociate from the channels leading directly to the decline of the modified current.

Since VTD binds rapidly to unmodified channels during a depolarization and decays very slowly upon repolarization, repetitive depolarizations in the presence of VTD leads to a cumulative increase of the magnitude of the VTD-modified permeability and a concomitant decrease in the unmodified permeability (Sutro, 1986; Rando 1987*b*). After a pulse train, the unmodified permeability recovers to its original value. The time course of this recovery is thus the time course of conversion of VTD-modified channels to unmodified channels. If the slowly decaying VTD-modified permeability (as in Fig. 6*A*) is a result of the dissociation of VTD from the channel, the time course of the decay should be identical to the time course of the recovery of the unmodified current after a pulse train. The following experiments test this hypothesis and show that it is incorrect. Rather, the data suggest that the decay of the VTD-modified permeability is a result of the conversion of modified channels from a conducting to a nonconducting state.

Nodes were stimulated at 10 Hz in the presence of VTD until a steady state was achieved. This resulted in an increase of the VTD-modified current and a concomitant reduction of the peak (unmodified) current. At increasing time intervals after the end of the pulse train, a single test pulse was given to assay the recovery of the peak current (Fig. 9*A*). Fig. 9*B* shows the time course of the recovery of the peak current (P_{Na}) and the decline of the VTD-modified current (P_{Na}^{VTD}) on the same graph. Long after the VTD-modified current had completely decayed (>12 s), the amplitude of the peak current continued to increase. Thus, the decline of the VTD-modified current cannot be equated with the dissociation of VTD from the channels. It should be noted that, in the absence of VTD, stimulation of a nodal membrane at 10 Hz results in no change of the peak current amplitude.

The recovery of the unmodified permeability after repetitive depolarizations as shown in Fig. 9*B* occurred in two phases. The time course was found to follow the sum of two exponential processes. The time constant of the more rapid recovery was 10.2 s; that of the slower recovery was 40.0 s. In a total of five such experiments, the rapid time constant averaged 9.4 ± 1.9 s and the slower time constant averaged 36.4 ± 3.9 s. The contribution of the slower component was variable from experiment to experiment and made up between 12 and 31% of the recovery of the peak current. These data suggest that the conversion of VTD-modified channels to unmodified channels occurs by two distinct processes.

In muscle, repetitive depolarization leads to an increase in the VTD-modified permeability to a maximum value, then it declines with continued stimulation (Sutro, 1986). From this data, it was suggested that there is a slow inactivated state that VTD-modified channels may enter with repetitive pulsing. More channels could be driven to this slow inactivated state with higher pulse frequencies, longer pulse durations, and higher VTD concentrations (Sutro, 1986). This phenomenon was barely detectable in nerve (Rando, 1987*b*). When nerve was stimulated at 40 Hz for

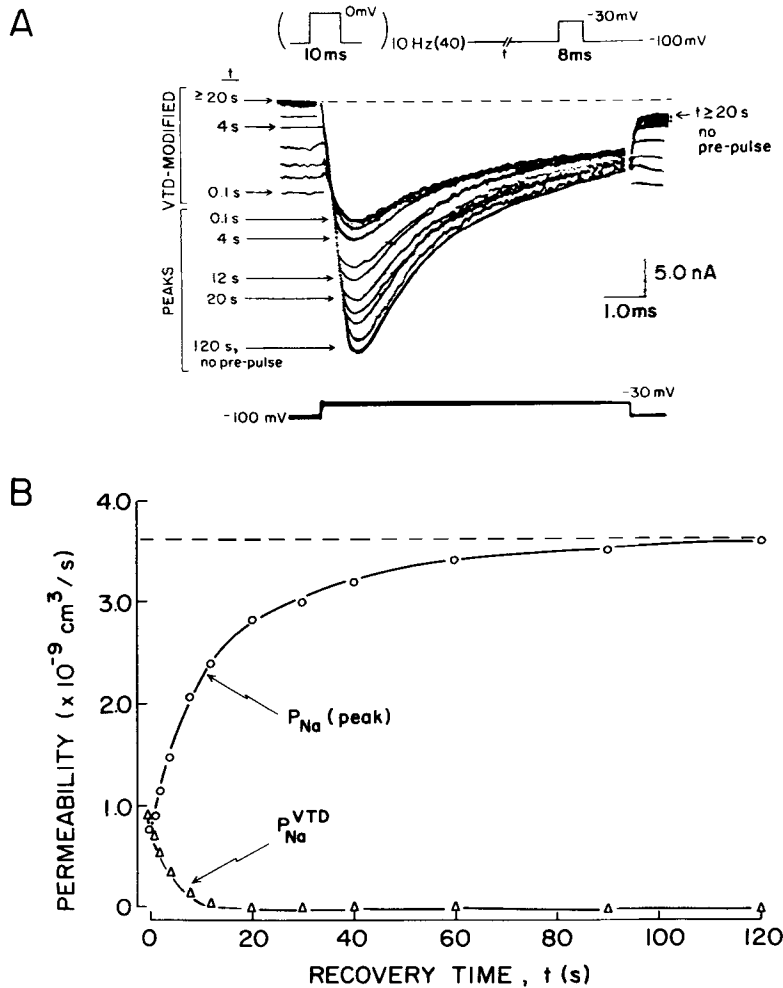


FIGURE 9. The relationship between the slow decay of the VTD-modified current and the dissociation of VTD from Na channels. (A) A node was stimulated at 10 Hz for 40 pulses to drive many channels to a VTD-modified state. The return of channels to an unmodified state was then assayed by a single test pulse at variable intervals after the pulse train and measuring the amplitude of the peak current. The traces are the responses to the test pulses. Indicated on the left and right are recovery times corresponding to certain modified and unmodified (peak) current traces. (B) The modified and unmodified (peak) currents shown in A were converted to permeabilities and the values of those permeabilities are plotted as a function of recovery time after the high frequency stimulation. The peak permeability increased from a minimum value (immediately after the train) to its initial value before the pulse train (dashed horizontal line) over ~ 2 min. The values of the peak permeabilities were adjusted for the variable contribution of the VTD-modified permeability during the test pulse. The modified permeability decreased from its maximal value (immediately after the train) to zero in ~ 12 s. The values of the modified permeability were increased by a factor of 1.33 to adjust for voltage-dependent gating and instantaneous rectification at -100 mV (see Fig. 3), both of which reduced the apparent modified permeability.

12 s, the VTD-modified permeability at the end of stimulation had decreased by <5% of the maximal value, whereas in muscle the permeability declined by nearly 50% (see Sutro, 1986 and Fig. 3). Furthermore, the experiments in nerve were done in four times the VTD concentration.

Nonetheless, to be sure that the results of Fig. 9, in particular the delayed and biphasic nature of the recovery of the unmodified permeability, were not due to this slow inactivation process, alternative stimulation parameters were used. With parameters of 1 Hz for 15 s or 10 Hz for 1 s, the amplitude of the modified current was smaller than in the experiment in Fig. 9. That is, fewer channels had been driven to a modified state. Nevertheless, after the cessation of the stimuli, the peak current recovered in two phases just as in Fig. 9, and the time constants of both processes were always similar to those with the stimulation parameters used in Fig. 9. The range of the slow time constants was 23–42 s for stimulation at 1 Hz to steady state ($n = 3$), and 28–37 s for stimulation at 10 Hz for 1 s ($n = 3$). Thus, it does not appear that the prolonged recovery of the peak current seen in Fig. 9 is due to the stimulation parameters of that experiment (10 Hz for 4 s).

Another interesting aspect of Fig. 9 is that, when a steady state was achieved by repetitive stimulation, the peak permeability was reduced by $\sim 2.85 \times 10^{-9} \text{ cm}^3/\text{s}$ (79%), whereas the corresponding VTD-modified permeability equalled $0.91 \times 10^{-9} \text{ cm}^3/\text{s}$ (or 25% of the unmodified peak permeability). The modified permeability accounted for only about one-third of the “missing” peak permeability. This would suggest that, compared with an unmodified channel, a modified channel has a smaller single channel conductance, a lower probability of being open at -30 mV , or both.

DISCUSSION

The rapid and slow gating of VTD-modified Na channels described here can be studied essentially independently because the time constants of the two processes differ by three to four orders of magnitude, depending on the membrane potential. To be able to study both processes in the same tissue is useful for comparisons with other systems in which rapid or slow effects of VTD have been described.

The Activation of VTD-modified Channels

The gating properties of VTD-modified channels in frog muscle fibers have been described by Leibowitz et al. (1986). The results presented here show that there are qualitative differences between the gating characteristics of such channels in nerve and muscle. One significant difference was that in nerve the time course of opening and closing of VTD-modified channels was well modeled by a single exponential process; in muscle the processes were well fit by the sum of two exponential components. The rapid component in muscle was very similar to the single component in nerve. The slow component in muscle, with time constants in the range of tens of milliseconds (Leibowitz et al., 1986), had no parallel in nerve. Whether this difference represents inherent differences between the Na channels in nerve and muscle, or differences between the tissues (e.g., Na channels in the T tubule system of muscle), remains to be determined.

Another significant difference is the sensitivity of the activation process to changes in membrane potential. In muscle, Leibowitz et al. (1986) reported that the slope of the curve of permeability vs. membrane potential was the same for VTD-modified channels as for unmodified channels. In nerve (Fig. 4), the curve for VTD-modified channels was clearly less steep than that for unmodified channels. It would be interesting to compare gating current studies in the presence of VTD in the two tissues to search for differences in the more fundamental aspects of the voltage-dependence of charge movement.

The Gating of VTD-modified Channels between a Resting and an Open State

VTD-modified channels gate between a nonconducting and a conducting (open) state in a voltage-dependent manner (Fig. 2). The nonconducting channels could be readily driven to the open state by a depolarization of the membrane (Figs. 2 and 3). This nonconducting state is, therefore, by traditional definition, a resting state. The transitions of the VTD-modified channels between the resting and open states followed an exponential time course, the time constant of which depended on the membrane potential (Fig. 5). These relaxations were modeled as the conversion of channels between a single resting state R^* , and a single open state O^* . In this model, the transitions between those states were governed by voltage-dependent rate constants, ℓ_1 and ℓ_{-1} , by the following scheme:



The values of ℓ_1 and ℓ_{-1} were derived using the time constants at -120 and -140 mV (Fig. 5), and the distribution of channels between the resting and open states at these same membrane potentials (Fig. 4 B):

$$\ell_1 = 151 \cdot \exp(0.0524 \cdot E_m) s^{-1} \quad (2)$$

and

$$\ell_{-1} = 0.006 \cdot \exp(-0.0321 \cdot E_m) s^{-1} \quad (3)$$

where E_m is the membrane potential. The solid curve in Fig. 5 shows the predicted relationship between the time constant of the relaxation and the membrane potential based on these rate constants where

$$\tau = \frac{1}{\ell_1 + \ell_{-1}} \quad (4)$$

The model was then applied to the voltage dependence of the distribution of channels between resting and open states. The solid line through the points for VTD-modified channels in Fig. 4 B is drawn according to the voltage-dependent rate constants above, where the fractional activation (A) is derived by the equation:

$$A = \frac{1}{1 + (\ell_{-1}/\ell_1)} \quad (5)$$

The model accurately describes the voltage dependence of the activation of VTD-modified channels.

One aspect of the data that is not included in the model is the fraction of channels that remained open at -200 mV, which is depicted graphically in Fig. 4 A. This may represent a distinct population of channels that do not undergo this gating reaction. Alternatively, this phenomenon may be indicative of a second modified open state, which is stable at very negative membrane potentials and which a fraction of channels enter instead of R^* . This point needs further investigation.

The Slowly Developing VTD-modified Permeability

The studies of the slow modification of Na channels by VTD extend the work of Ulbricht (1969, 1972*a, b*) and Leicht et al. (1971*a, b*) in which slowly developing currents in the presence of VTD were described. The importance of examining this slow modification lies not only in the fact that it is a second pathway by which a VTD-modified permeability may develop, but also because all of the effects of VTD on resting tissues are probably a result of this slow process. The depolarization of nerve and muscle cells and the increase of Na permeability of cells in tissue culture by VTD occur over many seconds or even minutes (Ulbricht, 1969; Ohta et al., 1973; Catterall, 1975; McKinney, 1984; Rando et al., 1986). These rates can be increased by electrical stimulation (Ulbricht, 1969; Rando et al., 1986), which probably brings into play the rapid process of VTD binding to open channels. Most studies of tissue culture cells are done in the absence of any external stimulation and thus the VTD effects most likely occur through the slow pathway. The extrapolation from VTD-induced changes of Na permeability of tissue-culture cells to the biophysical properties of VTD-modified Na channels is strengthened by an understanding of the underlying process that leads to those permeability changes. Garber and Miller (1987) used the planar bilayer technique to study the permeation properties of VTD-modified channels. In that technique, the artificial membrane is often held at depolarized potentials for many seconds between individual openings of VTD-modified channels. The kinetics of these openings of VTD-modified channels may also be governed by this slow process.

From the experiments presented, it is not obvious how the binding of VTD relates to the development of the slow permeability. In the model of Ulbricht (1969), it is presumed that VTD is associated with channels at rest, and those channels activate slowly during prolonged depolarizations. If that model was correct, then one would expect the unmodified permeability to be reduced in the presence of VTD. This is not what is observed (Fig. 1). If there is a binding of VTD to channels that then leads to a slowly developing current, that binding must occur after the activation of unmodified channels. I propose that the slow permeability develops by the binding of VTD to channels in the fast inactivated state, and that it is these modified, inactivated channels that then slowly open (see below). For prolonged depolarizations, the fast inactivated state is a transient state as channels progress to slow inactivated states. Thus, like the open state for the rapid binding of VTD (Sutro, 1986; Rando, 1987*b*), the transient nature of the state of the channel to which VTD preferentially binds limits the extent of the modification. Another possibility is that the slowly developing current arises from the slow reopening of inactivated channels and the

binding of VTD to those open channels. However, an argument is presented below that the slow development and slow decay of the VTD-modified current arise from the conversion of VTD-modified channels between an open and an inactivated state. The binding of VTD to the fast inactivated state of the channel may be the simplest hypothesis that is consistent with the data to explain the slowly developing current.

*A Reinterpretation of the Decay of VTD-modified Currents:
The Inactivation of VTD-modified Channels*

The decay of the VTD-modified current after a brief depolarizing pulse has been called a "tail current" because it is a decaying current that appears after returning the membrane to the holding potential at the end of a test pulse. For unmodified channels, the decay represents the "deactivation" transition of channels from an open to a resting state. However, there is a second process, namely inactivation, by which channels pass from an open to a nonconducting (inactivated) state while the membrane is maintained at a constant potential. I propose that the decay of VTD-modified currents, as shown in Fig. 6, represents the inactivation of VTD-modified channels. This postulate is based on the traditional distinction between resting and inactivated states.

Both resting and inactivated states are nonconducting states of the Na channel, distinguished by the kinetics of their transitions to open states. By convention, a resting closed Na channel is one that can open rapidly and with high probability in response to depolarization; an inactivated Na channel is one that opens extremely slowly and with low probability upon membrane depolarization. Using these criteria, the following argument leads me to conclude that VTD-modified channels pass from an open state to an inactivated state as the modified current decays. After the train of depolarizations illustrated in Fig. 9, ~80% of the channels were modified as judged by the reduction of the unmodified current. The VTD-modified current decayed completely within 20 s after the train, thus any channel still modified at that time would have been in a nonconducting state. This nonconducting state must be an inactivated state since, as shown in Fig. 4, VTD-modified resting channels would tend to open at -100 mV. It seems most likely that this inactivated state is a VTD-modified inactivated state and that the slow recovery of the peak, unmodified current represents the slow unbinding of VTD. Since inactivated states, whether modified by VTD or not, are physiologically "silent," it would seem that biochemical experiments would best answer the question of the rate of VTD disassociation. If, however, this hypothesis is correct, then VTD-modified currents decay because modified channels undergo a transition from an open state to an inactivated state.

This inactivation of VTD-modified channels is not simply a modification of normal fast inactivation of unmodified channels; it is a process with no known analogue in the kinetics of gating of unmodified channels. VTD-modified channels inactivate orders of magnitude more slowly than unmodified channels, and the open state is favored at more negative potentials for VTD-modified channels. This is the opposite voltage dependence to that of unmodified channels. In fact, this voltage dependence led Ulbricht (1969) to conclude that this slow process represented a modification of the activation process of unmodified channels. However, the reversal of

inactivation at positive potentials is not unprecedented in the literature of Na channel physiology and pharmacology. In the presence of *Leiurus* scorpion α -toxin, increasingly positive pulse potentials produce Na currents with less inactivation at steady state in the node of Ranvier (Wang and Strichartz, 1985). In the squid axon, in the absence of any neurotoxin, Chandler and Meves (1970) also found a reduction of steady state inactivation of the Na current with very positive depolarizations.

It is very important to distinguish the inactivation of modified channels discussed here from other definitions of the inactivation of VTD-modified currents. Leicht et al. (1971*b*) described an inactivation of VTD-modified channels in giant neurons of the snail *Helix pomatia*. This inactivation was a partial decay of the induced current during a depolarization of several seconds; this decay was not observed in my studies of frog nerve (Fig. 7). As discussed above, Sutro (1986) described an inactivation of VTD-modified channels that occurred during a series of depolarizing pulses. With pulsing, the magnitude of the VTD-modified current first increased to a maximal value, then slowly decreased with continued stimulation. This type of inactivation is barely detectable in the node (Rando, 1987*b*). Clearly, the inactivation processes described by both Leicht et al. (1971*b*) and Sutro (1986) are different phenomena from the inactivation described in this report.

The Gating of VTD-modified Channels between an Open and an Inactivated State

As suggested by the data in Figs. 6 and 8, the slow decay and slow development of the VTD-modified current may represent the forward and reverse direction of a single physiologic process. Thus, if the decay of the modified current represents channel conversion from an open to an inactivated state, then the slowly developing current would represent channel conversion from an inactivated state to an open state. The behavior of VTD-modified channels converting between an open state, O^* , and an inactivated state, I^* , can lead to an expanded model as follows:



The values of κ_1 and κ_{-1} were derived by assuming that, at most, 1% of the modified channels could be in O^* at -120 mV and that 8% would be in O^* at -80 mV. Then, using the time constants of the decay of the modified currents at these two potentials, the rate constants were defined by the equations:

$$\kappa_1 = 0.27 \cdot \exp(-0.0057 \cdot E_m) \text{ s}^{-1} \quad (6)$$

and

$$\kappa_{-1} = 1.73 \cdot \exp(0.048 \cdot E_m) \text{ s}^{-1}. \quad (7)$$

The time constant of this slow gating reaction was calculated as a function of membrane potential using the analogue of Eq. 4. The solid line in Fig. 10 is drawn according to the model and fits well the observed time constants over the range of -140 to -20 mV.

Similarly, the analogue of Eq. 5 was used to calculate the fraction of modified channels in the open state for the reversible inactivation gating. One of the predic-

tions of this model is that the membrane potential at which channels would be equally distributed between O^* and I^* is -35 mV. It was on this basis that the data in Fig. 7 B were normalized in Fig. 7 C. The VTD-modified permeability had not reached a maximal value at the most positive membrane potential that was possible to test, so the normalization was arbitrary. The data points were set on the scale such that a curve drawn by eye through them had a value of 0.5 (i.e., equal distribution of channels between O^* and I^*) at -35 mV. With the data normalized as such, the predicted values based on the model (Fig. 7 C, dashed line) followed the observed values, although there was some deviation at the more positive potentials. The calculated midpoint potential, -35 mV, is close to that assumed by Ulbricht (1969) in his studies of a slowly developing VTD permeability. He was able to maintain a node depolarized at potentials up to 60 mV more positive than the resting potential (i.e., ~ -10 mV), and he took the midpoint voltage to be ~ 30 mV by extrapolation.

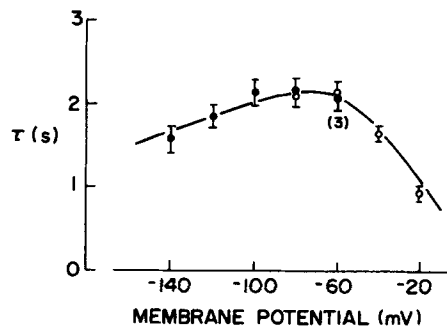


FIGURE 10. Time constants of the relaxation of modified channels between an open and an inactivated state. The solid circles represent time constants of the decay of modified currents as shown in Fig. 6. The open circles represent time constants of the development of the slow current as shown in Fig. 8. These time constants are combined on the same graph based on the model presented in the Discussion, and the solid line is drawn according to that model. All points represent the mean of determinations from five individual nodes except where the number of experiments is indicated in parentheses.

Dissociation of VTD from the Channel

Since the work of Ulbricht (1969), it has been known that the modification of Na channels by VTD can be promoted or reversed depending on the membrane potential. It seems implicit in that and subsequent studies that the reversal of VTD binding was equated with the slowly decaying (inactivating) modified current. That is, the assumption has been that the modified current decayed because VTD dissociated from the channel. The data in Fig. 9 refute this assumption. Clearly, long after the modified current completely inactivated there remained a significant proportion of modified channels.

Leibowitz et al. (1986) considered the slowly decaying VTD-modified permeability to represent the dissociation of VTD from the channel. An interesting result that they obtained is that at membrane potentials sufficiently negative to close many VTD-modified channels, the time constant of the current decay increases appreciably. Their conclusion is that VTD unbinds more slowly from closed channels than

from open channels. In light of the results presented here, however, I would interpret that result as showing that closed modified channels inactivated more slowly than do open modified channels. It is possible that instead of a single inactivated state as presented in the model, VTD-modified channels may proceed from one inactivated state to a second inactivated state. If the rate of dissociation of VTD from the two inactivated states were different, this could explain the biphasic nature of the dissociation of VTD from the channel as seen in Fig. 9. According to such a scheme, the time constants obtained from experiments such as those as in Fig. 10 (~9 and ~36 s) would be a function both of actual dissociation rates of VTD and of the kinetics of transitions between the two inactivated states. Measurements of the actual dissociation rates would best be done using biochemical techniques.

Many of the alkaloid neurotoxins, pyrethroids, and other lipid-soluble toxins have slow effects on Na channels (see Strichartz et al., 1987). It may be that all such modified channels gate slowly between conducting and nonconducting states along with the known rapid gating of channels modified by toxins such as batrachotoxin and aconitine. Although more attention has been paid to the rapid gating processes, further evaluation of the slow processes is likely to provide more information on the kinetics of the actions of these toxins on resting cells and on the behavior of modified channels in the planar bilayers.

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