

Electrophysiological Comparison of Insecticide and Alkaloid Agonists of Na Channels

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ABSTRACT Macroscopic currents in Na channels were recorded from adult frog skeletal muscle under voltage clamp as various toxins were added to the bathing medium. Veratridine, cevadine, and 3-(4-ethoxybenzoyl)-veracevine modified the Na channels in a use-dependent manner during depolarizations and held them open for 3, 2.4, and 1.2 s, respectively, at -90 mV. The three alkaloids modified channels in the same way. Activation gating was shifted about -100 mV by the modification, and reversible closing of the channels by strong hyperpolarizations slowed reversal of the modification. The synthetic insecticides deltamethrin, EDO, GH739, and GH414 also modified channels during depolarizations that opened channels. The modification lasted 3 s with deltamethrin, but only 3–5 ms with the others. Hyperpolarization speeded the shutting off of current in insecticide-modified channels, but no reversible activation gating could be demonstrated. The ionic selectivity, $P_{\text{Na}}/P_{\text{NH}_4}$, of channels was decreased by all of the toxins. This ratio was 0.11 in normal channels, 0.26 in insecticide-modified channels, and 0.7–1.6 in veratrum-alkaloid-modified channels. During use-dependent modification, the veratrum alkaloids reduced the total Na current markedly, while deltamethrin did not. Thus, alkaloid and insecticide modifications share many features but differ in how much the conducting properties of the pore are changed and whether the channel can close reversibly while the toxin remains bound.

INTRODUCTION

Agents modifying gating of ionic channels have been useful tools for understanding channel structure and function. One of the more diverse classes of modifiers of voltage-gated Na channels are the lipid-soluble activators or agonists, including batrachotoxin, aconitine, veratrum alkaloids, grayanotoxins, pyrethroids, and other classic insecticides such as DDT. They promote Na channel opening and induce repetitive firing, depolarizing afterpotentials, or maintained depolariza-

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tion of nerve and muscle (reviewed by Catterall, 1980; Khodorov, 1985; Hille et al., 1987).

For these lipid-soluble agonists, we still have neither a complete description of the effects on channel function nor a full understanding of how many binding sites on a single Na channel they interact with. Catterall's (1975*a, b*, 1977*a, b*) study of batrachotoxin, aconitine, veratridine, and grayanotoxin first showed competitive kinetics for their interactions and a heterotropic allosteric enhancement by scorpion toxin of the affinity for each agent. His findings are consistent with a common site of action for these four lipid-soluble agonists. On the other hand, other work shows potentiation by pyrethroids, rather than competition, of batrachotoxin, veratridine, and grayanotoxin actions (Jacques et al., 1980; Brown and Olsen, 1984). Such results suggest that at least one pyrethroid-binding site is distinct from that for the other agonists.

By electrophysiological criteria, the lipid-soluble agonists have classically been considered in three groups: (*a*) batrachotoxin and aconitine, (*b*) veratrum alkaloids, and (*c*) insecticides. The question whether their actions should be thought of as basically similar or fundamentally different can be addressed in part by electrophysiological criteria. Three clearly quantifiable electrophysiological effects had been described before we began our recent work. The first was a requirement for open channels to permit agonists to modify gating, reported for DDT and pyrethroids (Hille, 1968; Vijverberg et al., 1982, 1983) and for aconitine and batrachotoxin (Mozhayeva et al., 1977; Khodorov and Revenko, 1979). The second was the ability of modified channels to close and reopen rapidly when the membrane is hyperpolarized and then returned to the normal resting potential. Such reversible gating at negative potentials was first described with aconitine and then with batrachotoxin (Schmidt and Schmitt, 1974; Khodorov and Revenko, 1979). The third was an alteration of the permeability properties of the open pore, manifested as an overall reduction of the Na current, a decrease of the single channel current, and a decreased discrimination among such permeant ions as Na, K, and NH₄. Such changes of permeability had been demonstrated or suggested for batrachotoxin, aconitine, grayanotoxin, and veratridine (reviewed by Khodorov, 1985).

We have recently been investigating the electrophysiological effects of veratrum alkaloids and insecticides in more detail to explore further the similarities and differences among the three classic groups of lipid-soluble agonists. We observed additional parallels between the actions of veratridine and the other compounds (Sutro, 1986; Leibowitz et al., 1986*b*). We showed a strict requirement for open channels as the precursor of veratridine-modified Na channels and, with less rigor, the same requirement for insecticide-modified channels. We also found a dramatically shifted gating at negative potentials and a reduction of the macroscopic Na current in veratridine-modified Na channels. This article continues the comparison by looking for changes of ionic selectivity with veratrum alkaloids and insecticides and looking for evidence of shifted, reversible gating with insecticide-modified channels. As we had purified samples of several veratrum compounds and several insecticides, we could also make comparisons within these groups. Preliminary reports of some of this work have been presented (Leibowitz et al., 1986*a*; Schwarz et al., 1986; Hille et al., 1987).

METHODS

Preparation and Recording

The experimental protocol, described in detail by Sutro (1986) and Leibowitz et al. (1986b), was designed to record currents in Na channels of adult muscle. Pieces of *Rana pipiens* semitendinosus muscles were voltage-clamped by the method of Hille and Campbell (1976). When pulses were not being applied, the holding potential was -90 mV, which is near the resting potential of normal fibers. The muscle fiber fragment was mounted in a plastic chamber filled with a depolarizing intracellular solution (115 mM CsF, 5 mM NaCl, and 5 mM HEPES, pH 7.2) and allowed to equilibrate for 15–20 min after insertion of the agar bridges. Then the test (A) pool was exchanged for Ringer (115 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , and 4 mM MOPS [morpholinopropanesulfonic acid], pH 7.2) and recording was begun. The ionic selectivity measurements used an NH_4 Ringer prepared by replacing all the NaCl with NH_4Cl . The temperature was maintained at 9°C . The current signal was corrected for linear leakage and capacity currents using a manually adjusted analog transient generator, filtered with an active, four-pole Bessel filter, and digitized and stored by computer on magnetic tape for later analysis. All stimulus and digital sampling pulses were generated by a locally built digital stimulator, which was programmed by our LM^2 minicomputer (Kehl et al., 1975). Membrane current and voltage were also continuously recorded on a strip-chart recorder. Analysis programs included a nonlinear least-squares fitting routine based upon the Gauss method. In the text, average values are given \pm SEM. In many figures displaying mean values, error bars are shown representing ± 2 SEM. As is commonly done for this preparation (Hille and Campbell, 1976), we use permeability (P_{Na}) defined by the Goldman-Hodgkin-Katz current equation rather than conductance (g_{Na}) as a measure of the fraction of open Na channels at different potentials.

Toxins

Deltamethrin was kindly provided by Dr. Toshio Narahashi, Northwestern University, Chicago, IL, and the germine acetates by Dr. Werner Ulbricht, University of Kiel, Federal Republic of Germany (from samples originally in the collection of W. Flacke). All other toxins were purified or synthesized at the Division of Applied Organic Chemistry, CSIRO, Melbourne, Australia. The work on the purification and partial resynthesis of the alkaloids was carried out following the finding that most samples of veratridine, obtained either commercially or from other biological laboratories, were impure and contained other alkaloids of the ceveratrum type that could affect neurophysiological measurements. The pure veratridine was obtained by purification using droplet countercurrent chromatography (DCCC) of veratrine, followed by its analysis by a new high-performance chromatography (HPLC) method (Holan et al., 1984a). The veratridine was then hydrolyzed to veracevine and re-esterified with veratroyl chloride to pure veratridine. The synthesis and/or activities of the compounds were previously reported for EDO (Holan, 1971; Wu et al., 1980), the insecticidal oxime GH739 (Holan et al., 1984b), the dichlorocyclopropane insecticide GH414 (generic name, cycloprothrin; Holan et al., 1978, 1986), and the tetrafluorocyclobutane insecticide GH601 (Holan et al., 1983). The compounds tested are listed in Table I and shown in Fig. 1. Initially the drugs were dissolved in 100% ethanol. The alkaloids were then diluted into the appropriate Ringer solution and kept as stock solutions (100–500 μM), while the insecticides were diluted into Ringer immediately before each experiment. In all cases, the final ethanol concentration was $<1\%$. Upon addition to Ringer, the insecticides formed a milky suspension; therefore, the concentrations specified represent the added amounts, and the actual dissolved concentrations were unknown but saturated.

TABLE I
Lipid-soluble Agonists Tested

Compound	Molecular weight	Maximum tested concentration	Tail τ^*	Tail size
		μM		
Alkaloids				
Veracevine	509.3	500	23	Small
Veratridine	673.4	100	3,000	Large
Cevadine	591.4	100	2,440	Large
EBV	656.4	50	1,150	Large
GMA	567.3	500	1	Minute
GDA	625.3	250	1	Minute
Insecticides				
Deltamethrin	497.9	62.5	3,160	Large
EDO	324.2	100	5	Large
GH739	415	72.5	3	Large
GH414	478	22	None	None seen
GH601	494.1	140	5	Small

* Tail time constant at -90 mV.

Induction of the Drug-modified State

With veratridine, repetitive stimulation produces a drug-modified state of the Na channel lasting for several seconds at a holding potential of -90 mV (Sutro, 1986). The same was true for cevadine, 3-(4-ethoxybenzoyl)-veracevine (EBV), and the insecticide deltamethrin.

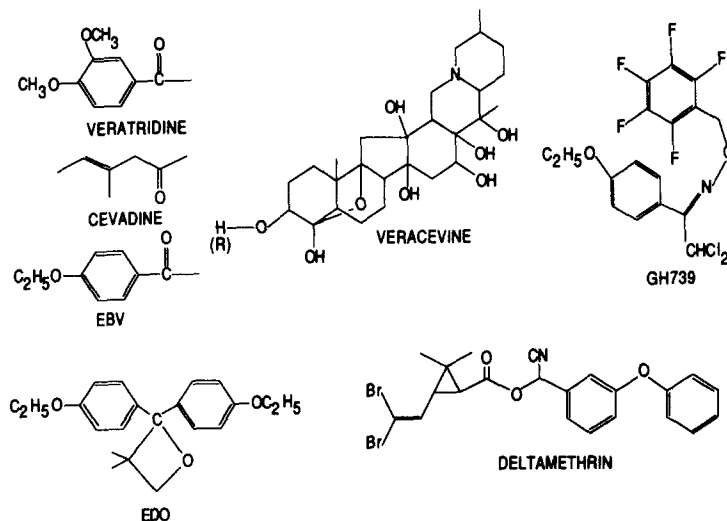


FIGURE 1. Structure of lipid-soluble agonists. GH414 has the same cyanophenoxycarbonyl group as deltamethrin but it is esterified with 1-(*p*-ethoxyphenyl)-2,2-dichlorocyclopropane 1-carboxylate.

In most experiments with these three toxins, we generated a population of modified channels, using a conditioning pulse train (often consisting of 10 10-ms depolarizing pulses to +6 mV delivered at ~3.7 Hz), and then studied the modified channels before they reverted to the unmodified state. Channels modified by the other compounds reverted to the normal state so rapidly that repetitive stimulation was not beneficial. For these agents, modified channels were studied in the tail following single depolarizing pulses; the channels were modified during the pulse, and remained transiently open during the tail when normal channels were closed.

RESULTS

Alkaloid-modified Channels

One goal of this work was to determine whether other purified veratrum alkaloids modify Na channels in the same way as veratridine does. The ability of veratrum alkaloids to modify gating is readily tested by looking for a persistent tail of Na current at -90 mV after a depolarizing pulse strong enough to open Na channels. A synopsis of the tail currents induced by the six alkaloids tested is given in Table I. Veracevine, germine-3-acetate (GMA), and germine-3,16-diacetate (GDA) produced small, or undetectable, tail currents and were not studied further. The naturally occurring alkaloids veratridine and cevadine, as well as the synthetic compound EBV, produced large, slowly decaying tail currents following 10-ms depolarizing pulses to -10 mV (Fig. 2A). The tail currents with these compounds often showed a small "hook," achieving their full size only after a couple of milliseconds at -90 mV. During repetitive stimulation, the tail currents grew larger and the peak current became smaller (Fig. 2A). One interpretation of the appreciable decrease in total current at the peak time (Leibowitz et al., 1986b) is that channels modified by veratridine, cevadine, or EBV conduct current less effectively than normal channels. At 100- μ M concentrations, cevadine was perhaps twice as efficacious as veratridine at inducing tail currents, and the fraction of channels modified in one pulse was twice as large, as if the apparent forward rate constant of the modification step were twice as high with cevadine.

All alkaloid-modified channels showed a gating process akin to normal activation gating but shifted to more negative membrane potentials. Cevadine- or EBV-modified Na channels shut rapidly after hyperpolarizations and reopened when the potential was returned to -90 mV, as has been reported for veratridine-modified channels (Sutro, 1986). We have examined the kinetics of these processes and compared them to those with veratridine. Fig. 3 presents observations on closing kinetics with a muscle fiber exposed to 100 μ M cevadine. A series of depolarizing conditioning pulses to +6 mV (not shown) resulted in a population of cevadine-modified Na channels manifested by the appreciable inward Na current shown at the beginning of the traces (where the membrane potential was -90 mV). Then when recording was begun, each subsequent sweep was preceded by a 10-ms depolarization to +6 mV (to maintain the population of modified channels) and contained a step to various hyperpolarizing potentials between -90 and -170 mV to look for channel closing. With cevadine, a few modified

channels closed spontaneously even at -90 mV, and the fraction of modified channels becoming closed increased with hyperpolarization. Upon "depolarization" to -90 mV after the hyperpolarization, most of the modified channels that had been closed reopened in a time-dependent manner. Such records were analyzed in terms of their closing kinetics as well as the steady state activation properties of the modified channels, as in our previous work (Leibowitz et al., 1986*b*). The reopening kinetics were not analyzed, but they clearly showed (Fig. 3) the paradoxical faster reopening after large hyperpolarizations that we had seen before with veratridine.

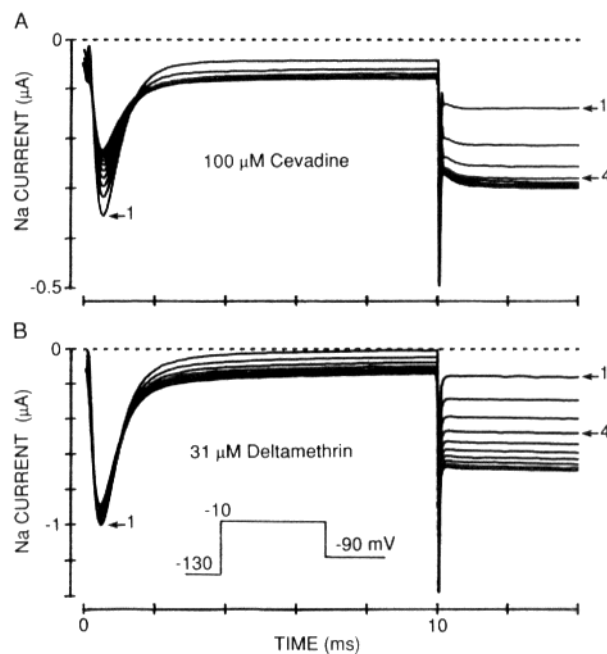


FIGURE 2. Use-dependent modification of Na channels with cevadine and deltamethrin. Rested single muscle fibers bathed in drug were held at -90 mV and stimulated with the following pulse sequence every 272 ms for 10 repetitions: -130 mV for 50 ms, -10 mV for 10 ms, and back to -90 mV. Currents during the first and fourth pulses are labeled "1" and "4." The dashed line is the current level at -90 mV before the pulse train. Time zero marks the beginning of the pulse to -10 mV.

The steady state fraction of channels closed by various test hyperpolarizations was determined from the current remaining when the potential was stepped back to -90 mV. This analytical approach avoided the need to correct for nonlinearities of the current-voltage relationship of modified channels. The fraction of open channels at each potential is shown in Fig. 4 for cevadine and EBV, together with the values for veratridine and unmodified Na channels taken from Leibowitz et al. (1986*b*). The lines for modified channels represent fits of a Boltzmann distribution. In comparison with normal channels, the activation curves for the three alkaloids are shifted markedly to hyperpolarizing potentials.

The midpoint for normal channels was -21 mV, while those for EBV- and cevadine-modified channels were -116 ± 4 mV ($n = 4$) and -110 ± 5 mV ($n = 5$), compared with -114 mV for veratridine observations from our previous article. The steady state activation curves for the three alkaloids are not statistically distinguishable.

At all potentials from -170 to -110 mV, the closing kinetics of alkaloid-modified channels exhibited both fast and slow components. A semilogarithmic plot of time constants for these two exponential components for cevadine and EBV is shown in Fig. 5. The solid line represents the values for veratridine taken from Leibowitz et al. (1986*b*). Qualitatively, the closing time constants for all of

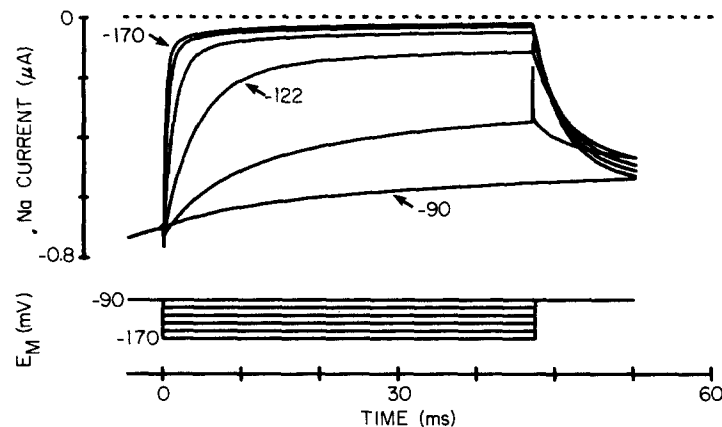


FIGURE 3. Voltage-dependent closing and reopening of cevadine-modified Na channels. The fiber was bathed in Ringer containing $100 \mu\text{M}$ cevadine and held at -90 mV. The experiments began with a conditioning train of 10 depolarizing pulses (not shown but as in Fig. 2) to produce a population of modified channels. Then during the measuring period, the depolarizing pulses were continued, but each was followed by a hyperpolarizing step to reveal gating of the modified channels. During the measuring period, the pulse pattern applied every 275 ms was: $+6$ mV for 10 ms, -90 mV for 5 ms, from -90 to -170 mV in intervals of 16 mV for 47 ms (to observe closing), and back to -90 mV. Traces recorded at -170 , -122 , and -90 mV are labeled. The analog signals were filtered at 10 kHz. Not the same fiber as in Fig. 2.

the alkaloids were the same. In other experiments, we found that the reopening time constants for cevadine-modified channels were indistinguishable from those for veratridine-modified channels (Leibowitz, M. D., and J. B. Sutro, unpublished data).

Veratrum alkaloids alter the ionic selectivity of Na channels. Permeability ratios for normal and alkaloid-modified channels were determined by comparing the reversal potential for currents in an Na Ringer with that in an NH_4 Ringer using a version of the Goldman-Hodgkin-Katz voltage equation (Hille, 1971). We chose NH_4^+ because it is the ion whose relative permeability is most profoundly increased by batrachotoxin, aconitine, and grayanotoxin (Khodorov, 1978; Moz-

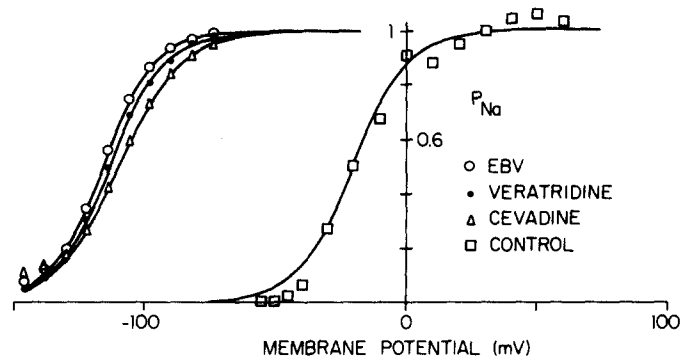


FIGURE 4. Shifted voltage dependence of activation gating with veratrum alkaloids. Control data (squares) show peak Na permeability at each test potential in a normal muscle fiber. The other symbols, for modified channels, are from "instantaneous" Na currents measured at -90 mV after 47-ms closing pulses to the indicated voltages (protocol as in Fig. 3): $100 \mu\text{M}$ veratridine (filled circles), $100 \mu\text{M}$ cevadine (triangles), and $50 \mu\text{M}$ EBV (open circles). The instantaneous currents are actually extrapolations to zero time using a straight line fitted to the first $150 \mu\text{s}$ of data at -90 mV. Extrapolation with an exponential-plus-offset function fitted to the first $700 \mu\text{s}$ gave identical results. The smooth curves represent the function $\{1 + \exp[(E - E_{0.5})/k]\}^{-1}$. The fitted values of the midpoint potential $E_{0.5}$ and slope factor k in millivolts are: -110 and 11.7 (cevadine, $n = 5$), -114 and 10.6 (veratridine, $n = 6$), and -116 and -9.7 (EBV, $n = 4$). The curve for control is simply the veratridine curve shifted to a midpoint of -21.2 mV. Data and curves for control and veratridine are taken from Leibowitz et al. (1986b).

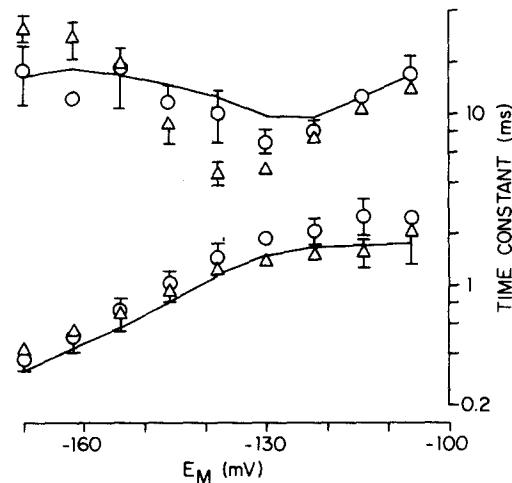


FIGURE 5. Voltage dependence of fast and slow closing time constants for cevadine, EBV, and veratridine. Current traces generated by the protocol of Fig. 3 were fitted with the sum of decaying exponentials. The resulting time constants are plotted semilogarithmically against the membrane potential. Veratridine: solid lines (from Leibowitz et al., 1986b). EBV: triangles ($n = 4$). Cevadine: circles ($n = 4$).

hayeva et al., 1977; Seyama and Narahashi, 1981). Possible artifacts from NH_4^+ permeability in K channels are expected to be small since *Rana pipiens* muscle has only tiny delayed K currents, which are further reduced both by the F^- and the Cs^+ of the internal solution. Reversal potentials for normal Na channels were determined from peak currents before the addition of any drug. Modified

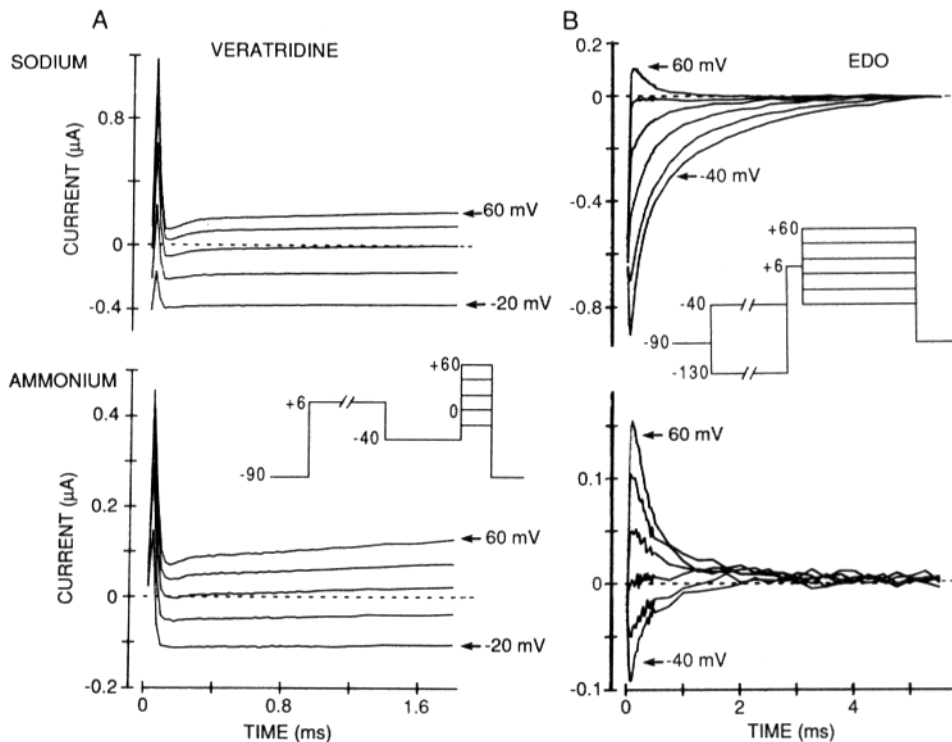


FIGURE 6. Ionic selectivity of agonist-modified Na channels measured from the reversal potential of tail currents. (A) Tail currents with $100 \mu\text{M}$ veratridine at -20 , 0 , 20 , 40 , and 60 mV recorded in Na Ringer and NH_4 Ringer. Modified channels were first induced by a train of depolarizing pulses at 4.5 Hz . During the measurement period, the pulses were 10 ms at $+6 \text{ mV}$ to maintain modified channels, 5 ms at -40 mV to check that the number of modified channels was constant, and 2 ms at various potentials to measure the tail. The zero-current line corresponds to the holding current level at -90 mV before the pulse train was applied. Time zero is the moment of stepping from -40 mV to the test potential. (B) Difference tail currents with $100 \mu\text{M}$ EDO at -40 , -20 , 0 , 20 , 40 , and 60 mV . The pulse sequence is described on pp. 87–88. Time zero is the moment of stepping from the brief inducing pulse ($+6 \text{ mV}$) to the tail test potential. The insets show the pulse protocols used.

channels were assayed as tail currents, recorded as in Fig. 6A and extrapolated to zero time by fitting a straight line to the points between 0.32 and 2 ms . Such permeability ratios are shown for control Na channels ($P_{\text{NH}_4}/P_{\text{Na}} = 0.11$, $n = 16$), as well as for channels modified with veratridine (0.67 , $n = 5$), cevadine (0.73 , $n = 3$), or EBV (1.57 , $n = 1$) (Fig. 7). In all cases, alkaloid-modified channels had

a significantly higher relative NH_4 permeability than control channels. The EBV-modified channels may be more permeable to NH_4 than to Na.

As in our previous article, we will identify the decay of the tail current at -90 mV with the dissociation of the agonist-receptor complex. While its several-second time course is clearly different from the millisecond time course of voltage-dependent gating, the two processes do interact. Hyperpolarization not only closed modified channels, but it also slowed the dissociation of the alkaloid agonists. The voltage dependence of the unbinding time constant for cevadine and EBV was determined electrophysiologically by the method used previously for veratridine (Leibowitz et al., 1986*b*). For potentials more positive than -100 mV (where modified channels have no gating), this requires only measuring the time constant of decay of the tail current. At more negative potentials (where gating closes modified channels quickly), the measurement requires adding

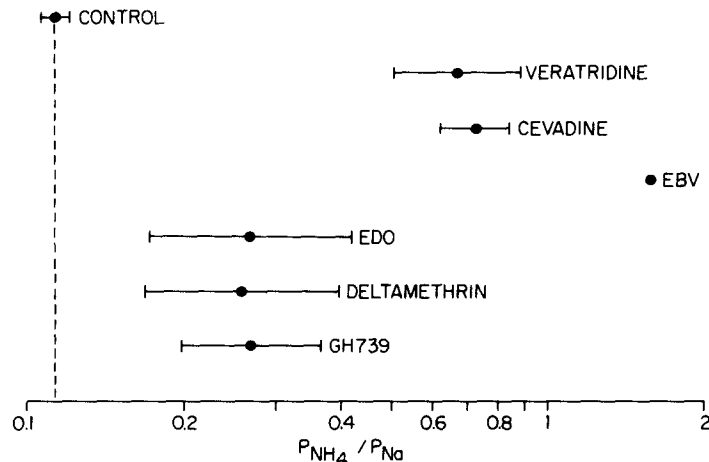


FIGURE 7. Relative NH_4 permeability of modified channels calculated from the change of reversal potential when Na is replaced by NH_4 .

occasional brief depolarizing pulses to reopen modified channels to test how many still remain. As with veratridine, these observations can be interpreted to mean that cevadine and EBV unbind more rapidly from open channels than from closed channels, and that the time constant for drug unbinding is voltage independent for either state (Fig. 8, A and B). We have extended the potential range over which cevadine unbinding was examined down to -240 mV and still find unbinding from closed-modified channels to be slow and voltage independent. The smooth curves in Fig. 8 are derived from the activation curves of Fig. 4 using the theory introduced before for veratridine. Namely, we assumed that the drug-receptor complex has one lifetime in the channel open state and another in the channel closed state. From this analysis, the complexes with EBV, cevadine, and veratridine have lifetimes of 1, 1.5, and 2.9 s in the channel open state and 9, 6, and 25 s in the channel closed state.

Insecticide-modified Channels

The second goal of this work was to compare Na channel modification by insecticides with that by veratridine. The tail currents induced by the insecticides tested are summarized in Table I. EDO, GH739, and deltamethrin produced

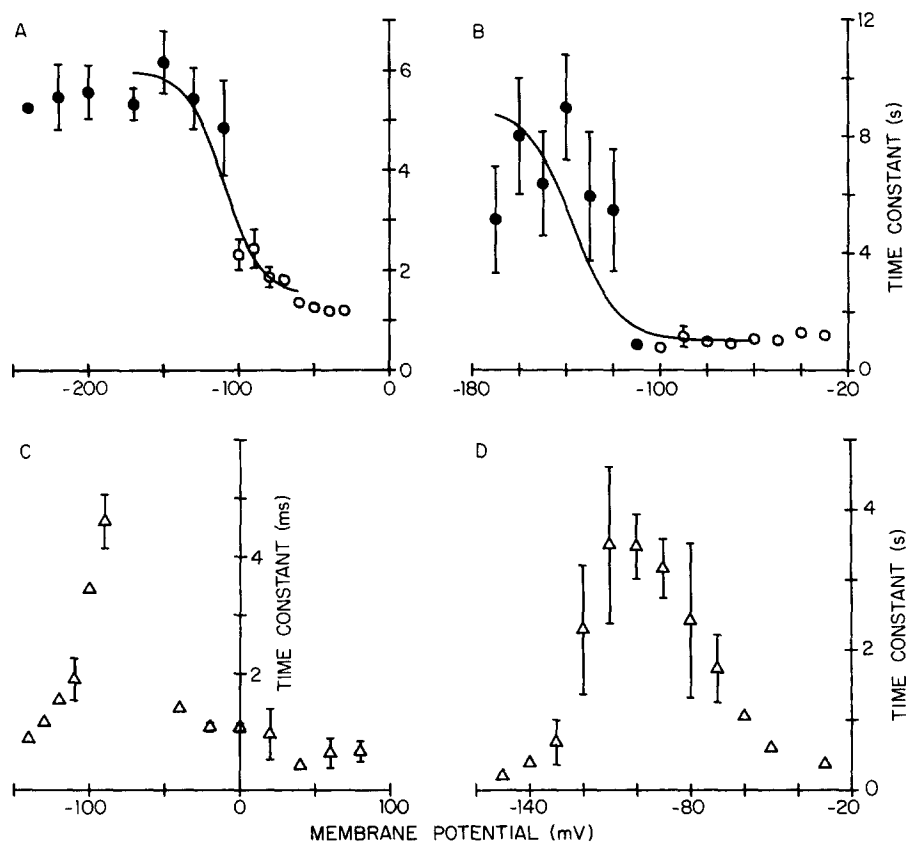


FIGURE 8. Voltage-dependent time constant for agonist unbinding defined electrophysiologically. Two protocols were used. For all symbols, the plotted value is the decay time constant of tail current, while the membrane is held at the indicated membrane potential. For the filled symbols, the population of remaining modified channels was assessed by brief depolarizing pulses to -74 mV as in Leibowitz et al. (1986b). (A) Cevadine. (B) EBV. (C) EDO. (D) Deltamethrin. The smooth lines in A and B are derived from the "activation" curves of Fig. 4 and the hypothesis that unbinding occurs with a longer time constant from closed channels than for open channels. The assumed time constants are 1.5 and 6 s for open and closed cevadine-modified channels and 1 and 9 s for EBV.

large populations of modified channels after depolarizing pulses and were studied extensively. The other insecticides produced very little modification or a modified state that was too brief to study. EDO and deltamethrin produced modified channel states that lasted for 4.6 and 3,200 ms at a potential of -90 mV. The

longer duration of deltamethrin modification made it possible to generate a large population of modified channels using conditioning pulse trains (Fig. 2*B*). Unlike the alkaloids, deltamethrin modification did not lead to much decline of the total current at the peak time, so the deltamethrin-modified channel presumably conducts better than the alkaloid-modified one, although still a little less than a normal channel. The effective forward rate constant of the modification reactions with EDO and GH739 was at least as high as for veratridine, since concentrations of 100 and 72.5 μM , respectively, gave severalfold-larger initial tail currents after a single depolarizing pulse than was seen with 100 μM veratridine. The modification reaction clearly depended on open channels, as the tail currents failed to appear if depolarizing prepulses had been used to inactivate normal Na channels. As with DDT, allethrin, and veratridine, the size of the induced tail of modified channels was directly proportional to the fraction of normal channels able to open in the test pulse.

We were unable to demonstrate reversible gating of insecticide-modified Na channels. Hyperpolarization did dramatically increase the rate at which the EDO-, deltamethrin-, or GH739-induced tail currents decayed (Fig. 9*A*); however, we have not observed reopening of the insecticide-modified channels upon the return to -90 mV after the hyperpolarization. With the normally long-lived deltamethrin-modified channels, it was possible to rule out channel reopening most convincingly. After a conditioning pulse train, the membrane was hyperpolarized to -170 mV for 45 ms, a potential at which the current flowing across the membrane fell quickly by $\sim 50\%$ and then continued to decline (Fig. 9*A*). The fall in current must result either from closing of channels in their modified form or from reversal of modification (drug unbinding). Fig. 9*A* shows that there was no rapid redevelopment of current at -90 mV once channels had closed. When the membrane potential was returned to -90 mV, the now attenuated current showed a small hook and then decayed slowly, rather than first growing back to its former large, negative value. Indeed, in another experiment to look at the full slow time course of the tail (Fig. 9, *B* and *C*), the tail was well described by the same single-exponential decay as in the traces preceding and following the test trace in which the membrane was not hyperpolarized. When scaled up, the traces were completely superimposable. Had there been significant slow reopening of modified channels during a test tail current, a single exponential would not have described the decay, and had there been significant fast reopening, it would have been seen in Fig. 9*A*.

The lifetime of open, insecticide-modified channels depends on the membrane potential. When channels closed during strong hyperpolarizing pulses, we could not use the method of small test depolarizations to assess how many of them were still modified since there was no reopening. Therefore, we could measure only the time constant of decay of the tail current itself. This parameter showed a bell-shaped potential dependence, falling off at depolarized and at hyperpolarized voltages (Fig. 8, *C* and *D*). We imagine but cannot prove that hyperpolarization may just drive the insecticide off the channel rather than closing the modified channel and trapping the drug.

Insecticides reduce the ionic selectivity of Na channels only a little. Deltamethrin-modified channels persist for seconds, and their ionic selectivity was studied

in exactly the same way as for the alkaloids. On the other hand, EDO and GH739, which produce transiently modified currents lasting for only a few milliseconds, could be studied in a different manner. Instead of having to rely on precise analog leak and capacity subtraction to isolate the transient tail

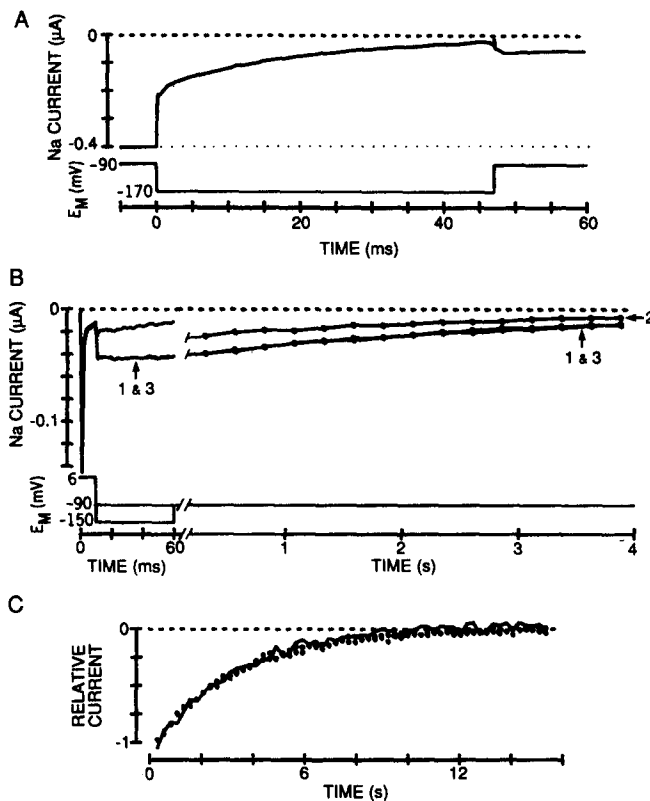


FIGURE 9. Lack of channel reopening after some deltamethrin-modified channels are closed by a hyperpolarization. Before the recording, a train of depolarizing pulses induced a population of modified channels. (A) A 47-ms hyperpolarization to -170 mV closed most of the channels, and on the return to -90 mV, no fast reopening was observed. (B) Three traces from another fiber with and without a 50-ms hyperpolarization to -150 mV to close about half the channels. The first and third trace (which are superimposed and indistinguishable) go directly to -90 mV and the second has the additional step to -150 mV. The tail is sampled only every 250 ms. (C) Superposition at slow speed of the tail currents at -90 mV from the experiment in B. The dots are from the two sweeps without a hyperpolarization and the solid line is the current after the hyperpolarization scaled up by 1.7 to match the size of the others.

currents, we could isolate them by digital subtraction of two digital records. The first record had a large 0.75-ms depolarization to open normal Na channels and to induce a population of modified channels, followed by a step back to the test potential to monitor the decay. The depolarizing pulse was preceded by a 90-ms

prepulse to -130 mV to remove resting inactivation of normal channels. The second digital record, made 1 s later, had the same sequence, preceded instead by a 90-ms modest depolarization (to -40 mV) to inactivate normal Na channels so that no modified channels would be induced in the strong depolarization. Subtraction of these two traces gave the difference tail currents (Fig. 6B). The slow component of their decay (from 0.5 to 5.5 ms) was fitted with a single exponential and extrapolated to time zero to determine the reversal potential. Deltamethrin, EDO, and GH739 gave permeability ratios ($P_{\text{NH}_4}/P_{\text{Na}}$) of 0.26, 0.27, and 0.27, respectively ($n = 3$ in each case) (Fig. 7). Thus, the selectivity change is much less than was found for veratrum alkaloids. The permeability ratio for insecticide-modified channels was not correlated with the duration of the modified channel state; deltamethrin-modified channels persist for orders of magnitude longer than EDO- or GH739-modified channels, and yet the three types of channel had indistinguishable permeability ratios.

Sutro (1986) suggested that veratridine-modified channels could enter a slowly inactivated state. He found that with rapid repetitive depolarizations, the tail current first grows by accumulation of modified channels, but then, later in the train, slowly diminishes again. The same phenomenon was seen when trains of depolarizations were applied to deltamethrin-treated fibers. Long trains of 3.5-ms depolarizations (to -10 mV) given at 20 Hz led to a visible secondary loss of the tail, and trains at 50 Hz led to a 50% secondary loss, as if 50% of the channels had become inactivated.

DISCUSSION

Comparison with Previous Work

The major goals of this work were (a) to probe the characteristics of alkaloid- and insecticide-modified Na channels further, particularly their permeability properties, and (b) to look for quantitative differences in the modifications that might suggest a difference in mechanism. Both classes of toxin produce modified channels that stay open at -90 mV, when normal, unmodified channels are shut. The quantitative characteristics of the modified channels are, however, drug specific. We have shown that channels modified by the efficacious alkaloids show markedly shifted steady state activation properties, significantly reduced ionic selectivity and conductance, and state-dependent unbinding of drug. The three insecticides that were studied intensively also hold channels open; however, they reduce ionic selectivity and conductance less, and the modified channel exhibits no gating and shows a bell-shaped, rather than a monotonic, relationship of modified-state lifetime to potential.

The active alkaloids used in this study differ only in the structure of the hydrophobic moiety esterified at carbon-3 of the veracevine nucleus. The three groups tested gave equivalent changes of Na channel gating with about the same large shift of activation and the same time constants of channel closing as those we had reported for veratridine (Leibowitz et al., 1986b). However, the moiety on C3 does have a modest influence on the lifetime of a drug-channel complex in the sequence veratridine > cevadine > EBV, and when there is no C3 moiety

(veracevine), one has a relatively ineffective compound that also produces a short tail current (Table I). The structure-activity relations apparently differ among preparations since GMA and cevadine, but not GDA, induce membrane potential oscillations in lobster axons (Honerjäger, 1973), while GMA, veracevine, and cevadine induce no Na flux in anemone toxin-treated neuroblastoma cells, and cevadine is reported even to be an antagonist of veratridine action there (Honerjäger et al., 1982). Veratridine is active in both preparations, and we could hardly distinguish cevadine from veratridine in frog muscle. Hence, the portions of the Na channel structure in the alkaloid receptor region are not fully conserved. (However, one has to be cautious in interpreting earlier studies that may not have used pure alkaloids.)

The insecticides we studied are diverse combinations of pyrethroid and DDT nuclei. Their actions share a striking difference from those of the veratrum alkaloids. Strong hyperpolarizations quickly turn off the persistent tail current that is present at -90 mV, and the tail current does not redevelop when the potential is returned to -90 mV. The steep voltage dependence of this turning off has already been reported for DDT, EDO, and pyrethroids acting on arthropod and squid axon (Lund and Narahashi, 1981, 1983; Wu et al., 1980), but in those studies the question of possible reopening was not asked. In our frog muscle assay, deltamethrin and EDO were quite effective, but GH414 and GH601 were not, whereas in anemone toxin-treated neuroblastoma cells, deltamethrin induced Na^+ tracer fluxes but the other three did not (Holan et al., 1985). Again there are selective differences among Na channels.

We found that veratrum alkaloids and insecticides change Na channel permeation but to different degrees. Veratrum alkaloids have the larger effect on ionic selectivity and on channel conductance. A reduction of selectivity has been reported for veratridine-modified channels of myelinated nerve (Naumov et al., 1979), but not, to our knowledge, unambiguously for insecticides. Small single channel conductances and reduced ionic selectivity have been demonstrated in rat muscle Na channels in lipid bilayers treated with veratridine (Garber and Miller, 1987), and a strong reduction of single channel conductance has been observed with N18 neuroblastoma cells (Barnes and Hille, 1987). No change of selectivity is reported with the insecticide tetramethrin in squid giant axons, a preparation that also is said to have no selectivity change with batrachotoxin (Yamamoto et al., 1986). Selectivity experiments with Na channel agonists are subject to several errors. Particularly if the Na channels are held open for a long time (veratrum alkaloids, deltamethrin, and tetramethrin), the internal ion concentrations can change each time the concentrations in the external medium are changed. The error, which depends on the flux-to-volume ratio, should be smaller for muscle fibers and squid axons than for nodes of Ranvier and should be negligible for planar bilayers.

Comparison with Other Lipid-soluble Agonists

Binding and competition studies with batrachotoxin, aconitine, grayanotoxins, and veratrum alkaloids suggest that they compete for a common receptor on the Na channel (Catterall, 1975a, b, 1977a, b, 1980; Catterall et al., 1981). From

the shape of the dose-response relationship with flux assays, Catterall (1980) suggests that a single bound agonist molecule suffices for activity. The electrophysiological observations show considerable quantitative differences between the effects of these agonists but a complete qualitative similarity. To the degree they have been studied, they all react preferentially with open channels, decrease inactivation, produce a modified, reversible activation gating that is slow, not very sigmoid in time course, and profoundly shifted to more negative potentials, reduce macroscopic conductance and single channel current, and decrease the ionic selectivity so that NH_4^+ becomes fairly permeant.

The lifetimes of the agonist-channel complexes range from a few milliseconds with veracevine to hours with batrachotoxin and aconitine. The lifetime correlates roughly with binding affinity and presumably reflects the free energy required to break the bonds with the channel. The extent of the shift of activation ranges from 20 to 30 mV with batrachotoxin to 100 mV with veratridine and does not correlate with binding affinity. It reflects the extra free energy needed to close the channel while agonist remains bound.

Binding and competition studies with pyrethroids place them in a different category. Pyrethroids potentiate the binding of batrachotoxin and the stimulation of flux by veratridine, grayanotoxin, and batrachotoxin (Jacques et al., 1980; Brown and Olsen, 1984), so it is presumed that pyrethroids bind to a different receptor. Similar studies have not been done with the DDT-type of insecticides. This difference would have been hard to anticipate from electrophysiological studies since, like the other agonists, insecticides react with open channels and hold Na channels open after a depolarization, slowing inactivation and reducing ionic selectivity at least a little. Only the lack of reversible gating of the modified channel looks qualitatively different. We cannot prove but do suggest that the insecticide molecule must leave the receptor before the channel can close and indeed that it is "squeezed" off the receptor when hyperpolarization is applied.

A Molecular Model

The phenomena can be described by a model discussed by Hille et al. (1987). The amino acid sequence of the Na channel main subunit shows four major homologous repeats that Noda et al. (1984) have suggested may undergo successive conformational transitions that lead to the open state of the channel. In our view, the last conformational change leading to the open state also exposes to the lipid bilayer a relatively hydrophobic region that is the alkaloid neurotoxin-binding site. Lipid-soluble agonists bind here, distorting the macromolecule enough to change the conductance and selectivity of the pore and stabilizing the open state enough to retard the inactivation process and prevent the usual quick closure at rest. A strong hyperpolarization will draw the binding site and the agonist into the macromolecule, permitting closure with drug remaining bound.

How would insecticides fit into this scheme? We are struck by the extraordinary similarity of insecticide actions to those of the alkaloids, despite the evidence that they bind to a different site. We suggest that their binding site may be homologous with that for the alkaloids, using similar portions of different repeats of the pseudosymmetric structure of the Na channel. Thus, this nearly equivalent

receptor also can stabilize the open state and change the pore; however, it differs in the chemical specificity that it offers and in its inability to withdraw into the closed macromolecule, while maintaining an adjoining pocket large enough for bound insecticide. Because insecticide binding to this site stabilizes the open pore, it also enhances the binding of veratrum alkaloids to their binding site and vice versa.

We thank Drs. T. Narahashi and W. Ulbricht for giving us compounds.

This work was supported by National Institutes of Health grants NS-08174 and RR-00374. M.D.L. was supported by National Research Service Award training grant NS-07097.

Original version received 15 October 1986 and accepted version received 12 January 1987.

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