Activation of *Drosophila* Sodium Channels Promotes Modification by Deltamethrin

Reductions in Affinity Caused by Knock-Down Resistance Mutations

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abstract *kdr* and *super-kdr* are mutations in houseflies and other insects that confer 30- and 500-fold resistance to the pyrethroid deltamethrin. They correspond to single (L1014F) and double (L1014F+M918T) mutations in segment IIS6 and linker II(S4–S5) of Na channels. We expressed *Drosophila* para Na channels with and without these mutations and characterized their modification by deltamethrin. All wild-type channels can be modified by <10 nM deltamethrin, but high affinity binding requires channel opening: (a) modification is promoted more by trains of brief depolarizations than by a single long depolarization, (b) the voltage dependence of modification parallels that of channel opening, and (c) modification is promoted by toxin II from *Anemonia sulcata*, which slows inactivation. The mutations reduce channel opening by enhancing closed-state inactivation. In addition, these mutations reduce the affinity for open channels by 20- and 100-fold, respectively. Deltamethrin inhibits channel closing and the mutations reduce the time that channels remain open once drug has bound. The *super-kdr* mutations effectively reduce the number of deltamethrin binding sites per channel from two to one. Thus, the mutations reduce both the potency and efficacy of insecticide action.

key words: insecticide • pyrethroid • *para* mutation • voltage gated

INTRODUCTION

Voltage-activated sodium channels provide selective and rapidly activating ion pathways required for action potential generation and propagation. The α subunit of these channels contains multiple binding sites for neurotoxins and therapeutically important drugs (Catterall, 1992). The molecular nature of many of these binding sites has been identified by systematic site-directed mutagenesis of the α subunit of mammalian sodium channels (Terlau et al., 1991; Ragsdale et al., 1994; Rogers et al., 1996; Cestele et al., 1998; Wang and Wang, 1998). Much less is known about the structure–function relationships of insect sodium channels because these proteins have only recently been cloned and the conditions for their functional expression have only recently been identified (Feng et al., 1995; Warmke et al., 1997). This heterologous expression provides new opportunities for structure–function studies because modified ligand binding sites can be identified by selecting for insects with resistance to neurotoxic ligands, especially synthetic chemicals that target insect sodium channels (Bloomquist, 1996; Narahashi, 1998). Comparative studies between mammalian and insect sodium channels can also provide insight into structure–function relationships because insect sodium channels are particularly sensitive to a number of neurotoxins such as pyrethroids (Narahashi, 1996; Vais et al., 1997; Warmke et al., 1997).

Pyrethroids are commonly used as insecticides in crop protection, animal health, and the control of insects that endanger human health. These insecticides combine high insecticidal activity with low mammalian toxicity and constitute >25% of the world insecticide market. The intensive use of pyrethroids over the last 20 yr has led to the development of resistance in many insect species (Sawicki, 1985) and this now represents the single most serious threat to their continued, effective use in many pest control programs. An important mechanism of resistance, termed knockdown resistance (or *kdr*),[1] confers cross resistance to the entire class of pyrethroids and is characterized by a reduced sensitivity of the insect nervous system to these compounds (Bloomquist, 1993). This type of resistance has been reported in many important pest species, but is best characterized in the housefly, where several vari-

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[1]*Abbreviations used in this paper:* ATX-II, isoleucine isoform of toxin II from *Anemonia sulcata*; *kdr* mutation, knockdown resistance mutation.

J. Gen. Physiol. © The Rockefeller University Press • 0022-1295/2000/03/305/14 $5.00
Volume 115 March 2000 305–318
Released online 28 February 2000
http://www.jgp.org/cgi/content/full/115/3/305

ants of *kdr*, including the more potent *super-kdr* factor, have been identified (Farnham et al., 1987). Evidence that resistance results from a modification of the sodium channel initially came from cross-resistance studies with sodium channel neurotoxins and binding studies that indicated a reduced affinity for pyrethroids on the sodium channel of *super-kdr* houseflies (Pauron et al., 1989). This was further supported by genetic mapping studies that showed close linkage between *kdr* resistance and the *para*-type sodium channel gene not only in the housefly (Williamson et al., 1993), but also in the tobacco budworm, *Heliothis virescens* (Taylor et al., 1993) and the German cockroach, *Blattella germanica* (Dong and Scott, 1994).

Molecular analysis of the full 6.3-kb coding sequence of the housefly *para*-type sodium channel identified two key amino acid substitutions in pyrethroid-resistant flies, L1014F in domain IIS6 and M918T in the IIS4–S5 linker (Williamson et al., 1996). L1014F is found in both *kdr* and *super-kdr* flies, while M918T is present only in *super-kdr* flies. Remarkably, the L1014F mutation has also been found in a wide range of pyrethroid-resistant strains of a number of other species, including cockroaches (Miyazaki et al., 1996; Dong, 1997), the lepidopteran *Plutella xylostella* (Martinez-Torres et al., 1997), Colorado potato beetles (Lee et al., 1999b), the aphid *Myzus persicae* (Martinez-Torres et al., 1999b), and the mosquitoes *Anopheles gambiae* and *Culex pipiens* (Martinez-Torres et al., 1998, 1999a). A different *super-kdr* mutation was identified in *Plutella*; i.e., a mutation in IIS5 corresponding to the housefly residue T929I (Schuler et al., 1998). To establish the role of these mutations in conferring resistance, we have incorporated the *kdr* and *super-kdr* mutations individually and in combination into cloned *Drosophila* sodium channels. The *Drosophila para* gene codes for a sodium channel α subunit and we have previously reported the expression of this protein in *Xenopus* oocytes alone and in combination with tipE, a putative *Drosophila* sodium channel accessory subunit (Warmke et al., 1997). We found that modification by permethrin, a type I pyrethroid (i.e., one lacking an α-cyano group), is >100-fold more potent for Para than for rat-brain type IIA sodium channels (Warmke et al., 1997; see also Feng et al., 1995). We now report a more extensive characterization of the modification of insect sodium channels by the type II (α-cyano) pyrethroid deltamethrin and show that the *kdr* and *super-kdr* mutations alter both the potency and efficacy of this insecticide. The *kdr* and *super-kdr* mutations also reduce the potency of cismethrin and cypermethrin to modify housefly sodium channels (Smith et al., 1997; Lee et al., 1999c). Another mutation in IS6 also reduces the affinity for permethrin (Lee et al., 1999a).

Modification of vertebrate Na channels by pyrethroids and other Na channel activators such as the plant alkaloids and halogenated hydrocarbons (DDT) is enhanced by electrical activity. This modification has generally been described with a "foot-in-the-door" model (Hille, 1992); i.e., the channels must open before the drug can bind and the drug must dissociate before the channels can close. Although this model can account for most features of the action of the alkaloids veratridine and batrachotoxin (Barnes and Hille, 1988; Zong et al., 1992), it has been less successful with the pyrethroids. Depending on the preparation, pyrethroids can increase, decrease, or leave unchanged the amplitude of sodium current, and they sometimes modify channels in the rested state (Chinn and Narahashi, 1986; Holloway et al., 1989; Ginsburg and Narahashi, 1993). Moreover, pyrethroids have been classified into two major subtypes, I and II, based on their electrophysiological effects (Gammon et al., 1981). For type I pyrethroids, typified by permethrin, there is a good correlation between insecticidal activity and the ability to induce electrical spiking activity in neurons after brief exposure. However, type II pyrethroids, typified by deltamethrin, are disproportionately weak at inducing spiking activity. This has led to the suggestion that some pyrethroids act at sites other than insect Na channels.

We find that deltamethrin effects on Para/TipE sodium channels are far more potent than those previously reported with vertebrate or marine invertebrate channels. This potency allowed us to examine the mechanism of action of deltamethrin at low concentrations of drug (0.1–10 nM). In this concentration range, the voltage dependence of sodium channel modification is simpler to describe and is generally consistent with a modified foot-in-the-door model. The *kdr* and *super-kdr* mutations reduce Na channel opening in the absence of drug by reducing the fraction of channels that open in response to depolarization (i.e, the mutations enhance closed-state inactivation). In addition, these mutations reduce the affinity of deltamethrin for Na channels and reduce the time that the channel remains open once drug has bound. Our studies suggest that the *super-kdr* mutations reduce the number of pyrethroid binding sites per channel from two to one. Thus, the mutations reduce both the potency and efficacy of insecticide action. Finally, we present a means of overcoming pyrethroid resistance.

MATERIALS AND METHODS

Plasmid Constructs and Mutagenesis

The *para* sodium channel cDNA construct (para 13-5) was as described previously (Warmke et al., 1997). The *kdr* (L1014F) and *super-kdr* (L1014F + M918T) mutations were introduced into this construct using the QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems). A *tipE* construct was generated by PCR amplification of the *tipE* gene coding sequence from *Drosophila melanogaster* cDNA. PCR primers were designed using the

published sequence (Feng et al., 1995) and the 1.4-kb fragment cloned into the pGH19 vector background of para 13-5 after removal of the 6.3-kb *para* insert with EcoR1 and Nru1. The resulting tipE construct (pGHtipE) was validated by DNA sequencing. Para and tipE cRNA transcripts were synthesized from Not1-linearized plasmid template using the T7 mMESSAGE mMACHINE kit (Ambion Inc.).

Materials

The isoleucine isoform of toxin II from *Anemonia sulcata* (ATX-II) was obtained from Calbiochem Corp. Racemic deltamethrin was obtained from Crescent Chemical (U.S. distributor for Riedel-de Haen); it was dissolved in ethanol and usually diluted 1,000-fold from a stock solution.

Oocyte Expression and Electrophysiological Measurements

Expression of sodium channel cRNAs in *Xenopus laevis* oocytes was performed as previously described (Warmke et al., 1997). In brief, cRNA transcripts of either para or mutant Na channels (concentration 1 μg/μl) were mixed with tipE transcripts and RNAase-free water, the final mixture having a 1:3 ratio by weight. Oocytes were injected with 50 nl of correspondent transcript solution, and used 2–5 d later.

Voltage-clamp experiments were performed using a CA-1 amplifier (Dagan Instruments). The bath solution was ND-96 consisting of (mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, adjusted to pH 7.5 with NaOH. In most experiments, the Na concentration of this solution was reduced by equimolar replacement of NaCl with *N*-methyl-d-glucamine to limit the maximal peak inward current to <1 μA, and thereby to achieve better voltage control. Experiments were performed at room temperature (21–23°C). Sodium currents were measured using a two-microelectrode voltage clamp and the holding potential was usually −90 mV; deltamethrin-induced tail currents were measured at −110 mV unless indicated otherwise. Voltage-measuring electrodes were filled with 1 M KCl and had resistances <1 MΩ. Current-injecting electrodes were filled with 0.7 M KCl plus 1.7 M K_3-citrate and had resistances <0.5 MΩ. Agar bridges to the bath electrodes contained platinized Pt wires and had resistances <7 kΩ. Data were acquired using the program Pulse (Instrutech Corp.), and most analyses were performed with the companion program Pulsefit. Linear leak and capacitive currents were subtracted with P/5 steps from −120 mV. Data were sampled at 50 kHz and filtered at 10 kHz, unless indicated otherwise.

RESULTS

Effects of kdr and super-kdr Mutations on Channel Gating

Previous studies with pyrethroids suggested that channel modification is promoted by opening (Leibowitz et al., 1987). Therefore, we first determined whether the *kdr* and *super-kdr* mutations reduce channel opening during depolarizations. Although several such mutations are known, only the L1014F single mutation and L1014F+M918T double mutation are studied in the present paper and, for simplicity, these are referred to throughout as kdr and super-kdr. In the absence of deltamethrin, channel gating appears to be little effected by these mutations (Table I and Fig. 1). The steady state voltage dependence of activation and inactivation and the rate of current decay during a depolarization are not different from control. However, large changes in channel availability due to these mutations are revealed by applying toxin II from *Anemonia sulcata* (ATX-II). This toxin dramatically slows inactivation of wild-type channels and increases the maximal sodium conductance ($G_{Na,max}$) approximately twofold (Fig. 1 A; Warmke et al., 1997). Fig. 1 A shows sodium currents measured during a strong depolarization with and without a maximally effective concentration of ATX-II. Note that the toxin nearly eliminates rapid inactivation and causes a large increase in peak inward current even for a strong depolarization that causes maximal activation. The extreme slowing of inactivation by ATX-II can be seen more clearly during a very long depolarization (Fig. 1 B). Thus, one can effectively eliminate inactivation with this toxin.

TABLE I

Effect of kdr and super-kdr Mutations in the Voltage Dependence of Para/TipE Na Currents

		Wild type	L1014F [i.e., L1014F (kdr)]	L1014F + M918T (super-kdr)
Activation	$V_{1/2}$ (mV)	−16.9 ± 1.4	−13.8 ± 1.1	−16.0 ± 1.3
	k (mV)	5.43 ± 0.40	6.03 ± 0.27	6.78 ± 0.32
Inactivation	$V_{1/2}$ (mV)	−43.7 ± 0.7	−38.2 ± 0.6	−42.9 ± 0.8
	k (mV)	4.52 ± 0.14	4.45 ± 0.22	4.67 ± 0.06
	τ_{decay} (ms) (−10 mV)	1.28 ± 0.08	1.34 ± 0.14	1.33 ± 0.09
	τ_{onset} (ms) (−40 mV)	13.9 ± 1.6	14.2 ± 1.7	8.3 ± 0.5
	τ_{recov} (ms) (−50 mV)	11.4 ± 1.1	7.6 ± 0.4	6.6 ± 0.9
ATX-II	$\Delta V_{1/2}$ (mV)	−5.6 ± 4.2	−4.7 ± 2.2	−4.9 ± 1.5
	$G_{Na,max}$ (fold increase)	2.2 ± 0.4	3.5 ± 0.5	4.9 ± 0.4

Fig. 1, D and G, shows the effects of ATX-II for the kdr and super-kdr mutant channels, respectively. The effects of toxin are similar to those with wild-type channels, but the increase in peak inward current is greater. This indicates that ATX-II causes even larger increases in $G_{Na,max}$ for channels with the *kdr* and *super-kdr* mutations (Fig. 1, C, F, and I). The effects of toxin on the voltage dependence of channel activation are shown in Fig. 1, E and H. The solid curves in Fig. 1 D indicate the best fit by a Boltzmann distribution assuming a linear single-channel current–voltage relationship. These fits indicate that the toxin increases $G_{Na,max}$ with little effect on the voltage dependence of channel activation. This toxin effect can be seen more clearly by converting the current–voltage relationship into conductance measurements (Fig. 1 H). Similar results were obtained in six other experiments with the L1014F mutation and three other experiments with the super-kdr double mutation (see Table I for a summary).

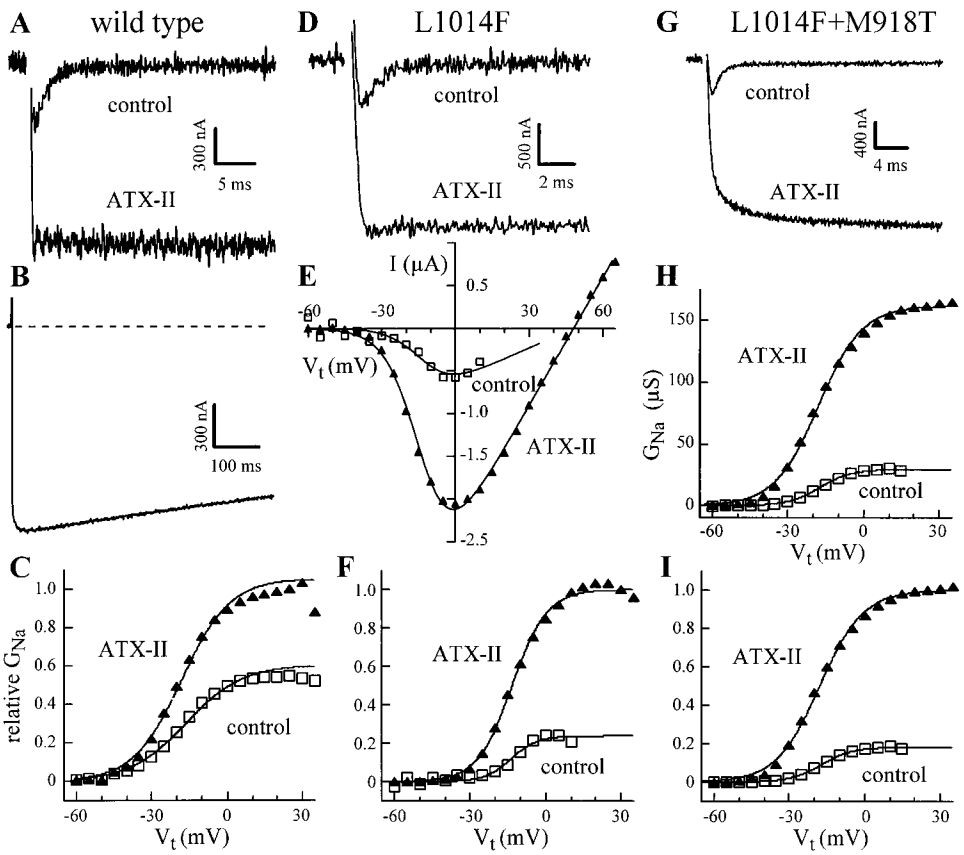

Figure 1. The *kdr* and *super-kdr* mutations reduce the availability of Na channels during a step depolarization. (A) Sodium currents measured at a test potential (V_t) of −10 mV with and without 0.5 μM ATX-II; blanking interval, 740 μs. (B) Sodium current measured in 0.5 μM ATX-II for the same preparation as for A. The test depolarization lasted 600 ms. (C) Relative G_{Na} plotted as a function of V_t with and without 100 nM ATX-II. In C, F, H, and I, $G_{Na,max}$ is calculated from the peak Na current assuming a linear current–voltage relationship and the curves are the best-fit Boltzmann distribution. Control: slope factor (k) = 9.03 mV, midpoint potential ($V_{1/2}$) = −19.1 mV; maximal sodium conductance ($G_{Na,max}$) = 5.33 μS; reversal potential = +44.0 mV. +ATX-II: k = 8.55 mV, $V_{1/2}$ = −19.4 mV, $G_{Na,max}$ = 9.71; reversal potential = +37.3 mV. In C, F, and I, the data are normalized by $G_{Na,max}$ in ATX-II to emphasize that toxin causes a greater percent change in $G_{Na,max}$ for kdr and super-kdr mutants than for wild-type channels. (D) Superimposed current records from an oocyte expressing the kdr Na channel with and without 1 μM ATX-II; V_t = 0 mV; blanking interval, 400 μs. (E) Peak sodium current plotted as a function of V_t with and without 1 μM ATX-II (same experiment as for A). Both curves assume a linear current–voltage relationship and are the best-fit Boltzmann distribution. Control: k = 6.58 mV, $V_{1/2}$ = −12.7 mV, $G_{Na,max}$ = 12.8 μS. It is assumed that the reversal potential for the Na current is the same as in ATX-II (+48 mV). +ATX-II: k = 6.74 mV, $V_{1/2}$ = −13.3 mV, $G_{Na,max}$ = 50.3 μS, reversal potential = 48. mV. (F) Relative G_{Na} with and without 1 μM ATX-II for a kdr mutant Na channel. Control: k = 6.58 mV, $V_{1/2}$ = −12.7 mV, $G_{Na,max}$ = 12.8 μS, reversal potential = +48.0 mV. +ATX-II: k = 6.74 mV, $V_{1/2}$ = −13.3 mV, $G_{Na,max}$ = 50.3 μS, reversal potential = +48.0 mV. (G) Sodium currents recorded at 0 mV with and without 1 μM ATX-II from an oocyte expressing super-kdr Na channels; blanking interval, 400 μs. (H) Peak G_{Na} plotted as a function of V_t with and without 1 μM ATX-II for a super-kdr Na channel. Control: k = 6.21 mV, $V_{1/2}$ = −17.3 mV, $G_{Na,max}$ = 29.4 μS, reversal potential = +47.0 mV. +ATX-II: k = 8.58 mV, $V_{1/2}$ = −17.9 mV, $G_{Na,max}$ = 161.8 μS, reversal potential = +47.7 mV. (I) Same data as in H normalized by $G_{Na,max}$ in ATX-II.

As for wild-type channels, the actions of ATX-II can be accounted for solely by slowing of inactivation and suggest that ATX-II inhibits inactivation from both open and closed states (Warmke et al., 1997). To emphasize the difference in response to ATX-II between wild-type and mutant Na channels, the conductance–voltage relationships are plotted with each data set normalized by $G_{Na,max}$ in the presence of ATX-II (Fig. 1, C, F, and I). Note that the midpoint and slope factor for all conductance–voltage relationships are similar, but that the fraction of channels that open with inactivation intact is greater for wild type than for the mutants. The nearly fivefold increase in $G_{Na,max}$ seen for the super-kdr mutant suggests that the mutations increase the likelihood that sodium channels inactivate without first opening, also known as closed state inactivation. Indeed, the mutations selectively increase the rate of inactivation for weak depolarizations (to −40 mV) that cause substantial inactivation but little channel opening (Table I and Fig. 2). For strong depolarizations, inactivation is primarily from open states and this rate is not effected by the mutations. Although the rate and amount of closed-state inactivation is increased by the mutations, the steady state voltage dependence of inactivation is little changed (Table I).

Modification by Deltamethrin Is Promoted by Channel Opening

Previous studies with vertebrate and marine invertebrate Na channels have shown that pyrethroids slow inactivation and deactivation and induce channel opening at more negative potentials than normal (Narahashi, 1996). Deltamethrin has similar effects on *Drosophila* Na channels, but the effects are more potent and the slowing of gating transitions is more extreme (Fig. 3). Unmodified Na channels close extremely rap-

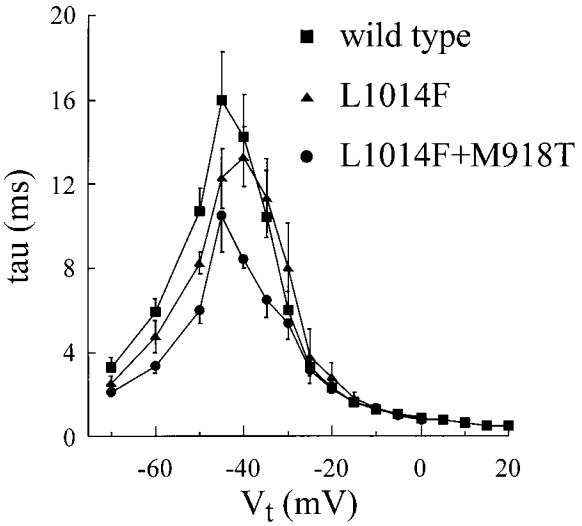

Figure 2. Onset of and recovery from inactivation is faster for the kdr and super-kdr Na channels. Three different pulse protocols were used. For $V \leq -50$ mV, symbols indicate the time constant for recovery from inactivation. For $-50 < V \leq -35$ mV, symbols indicate the time constant for onset of inactivation. Both sets of data were obtained by applying conditioning pulses of varying duration before the test pulse; in the case of recovery from inactivation, conditioning pulses were preceded by a preconditioning pulse to induce inactivation. For $V > -35$ mV, symbols indicate the time constant for onset of inactivation determined by fitting a single exponential to the decay of the current during a test pulse. Each data point represents the mean of at least four experiments and the error bars indicate SEM.

idly when the membrane is repolarized, but deltamethrin-modified channels remain open even at very negative potentials. The resulting insecticide-induced tail currents serve as a measure of modification. Fig. 3 A compares two tail currents obtained during application of 10 nM deltamethrin: one was induced by a 500-ms depolarization to 0 mV, the other was induced by a 100-pulse train of 5-ms depolarizations to 0 mV. Tail currents due to unmodified channels cannot be resolved due to the limited time resolution of a two-microelectrode voltage clamp of oocytes. Although the two-pulse protocols resulted in equivalent total durations of depolarization to 0 mV, the train of brief pulses produced a more pronounced tail current. This suggests that deltamethrin interacts with the open state of the wild-type Na channel.

Likewise, modification by deltamethrin is promoted by ATX-II, which increases channel opening by slowing inactivation (Fig. 3 B). The upper record shows the current during a 1-s depolarization with and without 100 nM deltamethrin. Even with this relatively high concentration, there is no channel modification. The lower record shows the same pulse protocol after adding 100 nM ATX-II. There is a small component of rapidly activating Na current also seen with ATX-II alone (see Fig. 1). The slow increase in current during the depolarization and large tail current are due to deltamethrin and indicate greatly potentiated modification. Thus, modification by deltamethrin is enhanced by an agent that increases occupancy of the open state at the expense of the inactivated state.

Fig. 3 C shows a detailed examination of the voltage dependence of modification by deltamethrin. The voltage dependence of channel opening was determined in 1 μM ATX-II (□). This toxin simplifies measurements of channel opening because it eliminates rapid inactivation and thereby facilitates measurements of activation over an extended voltage range. The oocyte was then exposed to deltamethrin and modification was induced with trains of depolarizations. The amplitude of the tail current measured at the end of the pulse train is plotted as a function of the conditioning voltage (V_c) used during the train (▲ and right-hand ordinate). The ordinates have been adjusted so that the maximal tail current coincides with maximal Na conductance. The close correlation between the voltage dependence of channel opening and that of pyrethroid modification indicates that channels must open before modification by deltamethrin can occur. The experiment shown is with Na channels containing the *super-kdr* mutations; these were easier than wild type to study because the deltamethrin-induced tail currents decay more rapidly. Similar results were obtained with wild-type channels.

Quantifying the Modification of Sodium Channels by Deltamethrin

Thus far we have used tail current analysis to measure relative levels of channel modification by pyrethroids. To quantify the effects of *kdr* and *super-kdr* mutations on the affinity of deltamethrin, it is necessary to have a measure of the fraction of Na channels modified by this ligand. If we assume that the single channel conductance is unchanged by deltamethrin, as is the case for mammalian sodium channels (Chinn and Narahashi, 1986; Holloway et al., 1989), then the fraction of modified channels is equal to $G_{Na,\delta m}/G_{Na,max}$, where $G_{Na,\delta m}$ is the conductance of deltamethrin-modified channels. If one also assumes a linear current–voltage relationship, then:

$$G_{Na,\delta m} = (\text{peak tail current})/(V_{tail} - V_{rev}), \qquad (1)$$

where V_{tail} is the voltage of the tail current measurement (typically −110 mV in our studies) and V_{rev} is the reversal potential. This equation is similar to that used for studies of mammalian sodium channels (Tatebayashi and Narahashi, 1994). There are two complications associated with the application of Eq. 1 to insect Na channels. First, previous studies assumed that all sodium channels are open at peak inward current during a strong depolarization. Our studies with ATX-II indi-

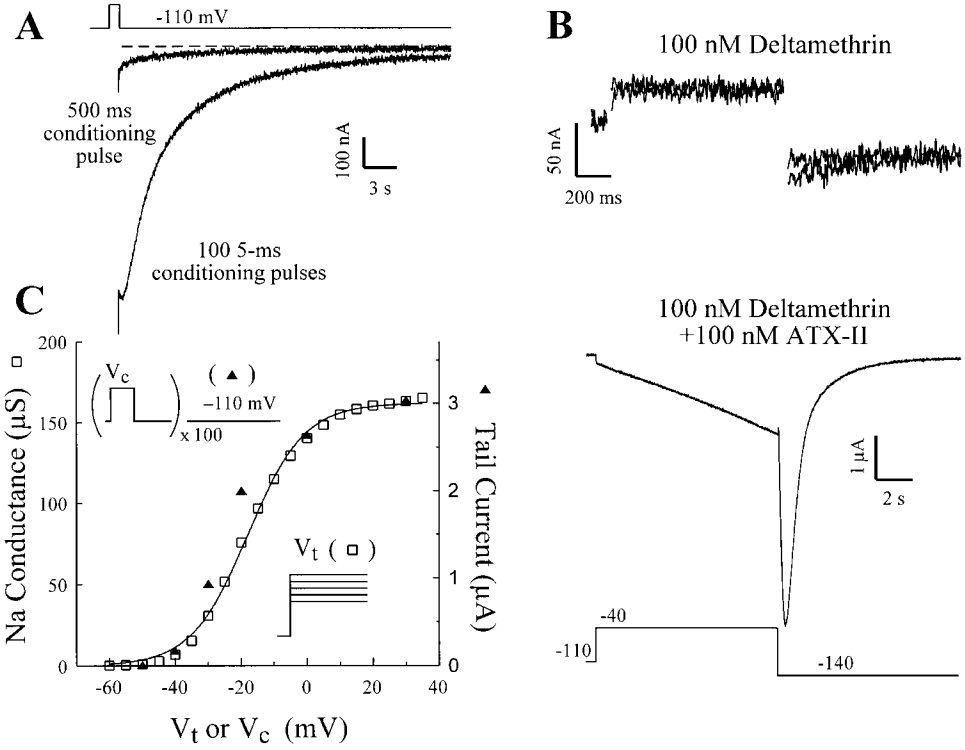

Figure 3. Modification by deltamethrin requires Na channel opening. (A) Trains of brief depolarizations are more effective than one long depolarization at producing modification by deltamethrin. Tail currents were measured at −110 mV after depolarizations to 0 mV using the indicated pulse protocol. The train of depolarizations was delivered at 66 Hz; 10 nM deltamethrin. (B) Deltamethrin induces large tail currents after a single long depolarization if inactivation is first slowed by ATX-II. The upper record shows superimposed current measurements with and without 100 nM deltamethrin alone. The voltage was stepped from −90 to −40 mV for 1 s and repolarized to −130 mV. Note that these records are at higher gain than below. The lower current record is offset. The pulse protocol is shown at the bottom. ATX-II causes a small rapidly activating current that was also seen with ATX-II alone. This is followed by a slowly activating current and, subsequently, a large tail current. The latter is not obtained with ATX-II alone (Fig. 1). (C) Tail current induced in 500 nM deltamethrin by trains of pulses increases with stronger depolarizations and the increase parallels the voltage dependence of channel opening. □ (left ordinate), peak G_{Na} for super-kdr mutant in 1 μM ATX-II. This toxin eliminates current decay during the trains of pulses so that peak current is proportional to the time integral of current. ▲ (right ordinate), amplitude of tail currents elicited after application of 100 conditioning pulses (5 ms at 66 Hz) for each conditioning voltage (V_c; see top inset for the tail current pulse protocol). The ordinates have been adjusted so that the maximal tail current amplitude coincides with maximal Na conductance.

cate that many insect Na channels do not normally open during a strong depolarization and that channel availability is altered by the *kdr* and *super-kdr* mutations (Fig. 1). Therefore, we increase the apparent $G_{Na,max}$ by the fold-increase due to ATX-II. As shown below, this still underestimates $G_{Na,max}$ because ATX-II is unlikely to increase the open probability to 1.

In addition, Eq. 1 assumes that the current–voltage relationship is linear at all voltages, but this has only been validated for $V_t > -40$ mV (Fig. 1). This assumption was tested by measuring the current–voltage relationship of tail currents induced by deltamethrin (Fig. 4). The voltage was ramped at 1 V/s before and after a train of test pulses in 1 nM deltamethrin. The plot shows the difference current from −120 to −50 mV; at more positive potentials the current–voltage relationship is linear (Fig. 1). Since the deltamethrin-induced tail current decays very slowly, this measurement indicates the single-channel slope conductance. The current–voltage relationship is highly nonlinear, perhaps due to voltage-dependent block by extracellular calcium (Yamamoto et al., 1984). Consequently, the effective driving force at −110 mV is ($V_{tail,eff} - V_{rev}$) or (−70 − V_{rev}) (mV). We could not use the same ramp protocol to determine current–voltage relationship for super-kdr sodium channels because the deltamethrin-induced tail currents decay too rapidly (see Figs. 5 and 7). We therefore assumed that $V_{tail,eff} = -70$ mV for both wild-type and mutant sodium channels. If we incorporate correction factors into Eq. 1 appropriate for insect Na channels, then we have:

$$\begin{aligned}&\text{fraction of drug-modified channels}\\&\quad = G_{Na,\delta m}/G_{Na,max}\\&\quad = [(\text{peak tail current})/(V_{tail,eff} - V_{rev})]/\\&\quad [(G_{Na,max}) \bullet (\text{ATX-II availability factor})]\end{aligned} \qquad (2)$$

where the ATX-II availability factor is the fold increase in $G_{Na,max}$ indicated in the last row of Table I and $V_{tail,eff} = -70$ mV.

The kdr and super-kdr Mutations Reduce the Affinity of Deltamethrin for Para Sodium Channels

Fig. 5 shows two protocols used to vary the amount of modification by deltamethrin. Fig. 5 (top) shows deltamethrin-induced tail currents measured after a train of brief test pulses of constant duration and with increasing concentrations of deltamethrin. The lower panels

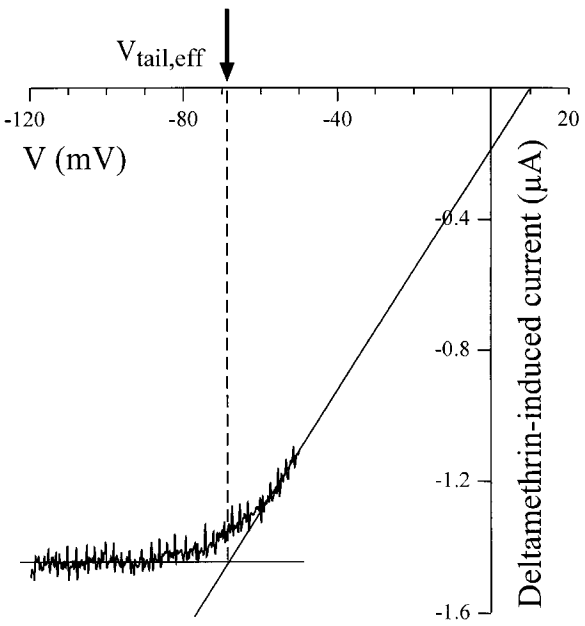

Figure 4. Instantaneous current–voltage relationship of wild–type sodium channels modified by 1 nM deltamethrin. The voltage was ramped at 1 V/s before and after a train of 200 pulses of 5-ms duration at 66 Hz. The record shows the difference between measurements made before and after the pulse train, and thus indicates the current induced by deltamethrin. A linear extrapolation from the data goes through the reversal potential determined in this oocyte before adding deltamethrin (V_{rev} is approximately +10 mV in saline with 20 mM Na). This extrapolation was necessary because at voltages more positive than −50 mV channels not modified by deltamethrin were also opened. The dotted vertical line indicates that the driving force on the sodium current at −110 mV is equivalent to that obtained with a linear I-V at about −70 mV.

show tail currents in a fixed concentration of deltamethrin and for pulse trains of increasing duration. The tail currents are easiest to understand for the *super-kdr* mutant (Fig. 5, E and F). For this mutant, the time course of the tail current is independent of the amount of modification and only the amplitude of the tail current increases with higher concentration of insecticide or longer pulse trains. This is the result expected for a 1:1 ligand–receptor binding reaction and was observed in studies of vertebrate sodium channels modified by pyrethroids or alkaloids such as veratridine (Zong et al., 1992; Song and Narahashi, 1996). Deltamethrin-induced tail currents through wild-type channels differ from those through L1014F + M918T double mutants in four principle ways (Fig. 5, A and B): (a) the tail currents decay much more slowly and are not described by a single exponential (note that the time scales in E and F are expanded ~10-fold), (b) the time course slows as the amount of modification increases, (c) the concentration–response relationship is much steeper, and (d) large tail currents are produced by much lower concentrations of deltamethrin. Tail currents through *kdr* mutants are intermediate between wild-type and *super-kdr* mutants in their time course and sensitivity to deltamethrin (Fig. 5, C and D). For wild-type channels, the very steep dependence of current amplitude on concentration and the variable time course of tail currents are inconsistent with simple models of 1:1 ligand–receptor binding and suggest higher order binding kinetics. According to this idea, the time course of tail current decay reflects the rate of drug dissociation and this rate is slowed by occupancy of multiple binding sites on each channel. In contrast, sodium channels with the *super-kdr* mutations behave as though there is only one binding site per channel. Additional tests of these ideas are presented below.

The time course of modification by deltamethrin is determined by applying trains of brief depolarizations of varying duration. The peak amplitude of the deltamethrin-induced tail current is converted into fractional modification using Eq. 2 and the percent modification is plotted as a function of pulse train length (Fig. 6). For the wild-type channel, modification by deltamethrin is effectively irreversible so that all channels can be modified by 3 nM insecticide after a long pulse train (Figs. 5 B and 6 A). In contrast, modification of the *super-kdr* mutant quickly saturates as the pulse train is prolonged, and even 5 μM deltamethrin modifies only 6% of the channels (Fig. 6 B). The solid curves are the best fit to an exponential rate of onset of modification. For both the wild-type and super-kdr channels, the onset of modification is well described by first-order kinetics. For the wild-type channel, we carefully looked for a sigmoidal onset rate predicted by a higher-order binding process, but there was no consistent evidence for this at 1–5 nM deltamethrin.

Although there are clear differences in the sensitivity to deltamethrin among the three constructs, it is difficult to assess the relative affinity for open channels by applying trains of test pulses. A larger fraction of the channels open for the wild type (as shown by studies with ATX-II described above) and the slower decay of wild-type tail currents allows for greater levels of modification as the pulse train is prolonged (compare Fig. 5, B with F, and 6, A with B). To distinguish between effects of mutations on binding affinity and those on channel gating, we determined modification by deltamethrin in the presence of maximally effective ATX-II during a single long depolarization (320 ms to 0 mV; Fig. 7). This protocol is an attempt to produce equivalent amounts of channel opening for all three constructs. The tail currents that are induced are qualitatively similar to those obtained in the absence of ATX-II: tail currents through the super-kdr channels decay relatively rapidly and the time course is little effected by the concentration of deltamethrin; tail currents through wild-type channels decay much more slowly, the time course slows as the con-

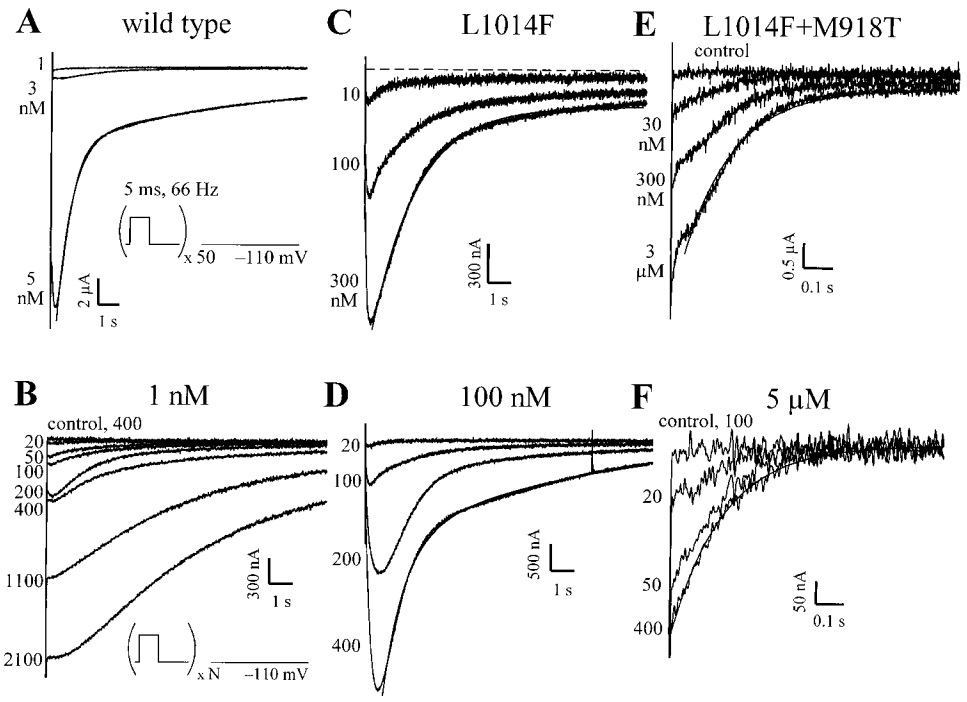

Figure 5. Deltamethrin-induced tail currents measured after trains of 5-ms depolarizations to 0 mV at 66 Hz. The left, center, and right columns show results for wild type, *kdr*, and *super-kdr* mutants, respectively. Note the expanded time scale in E and F. A, C, and E each show tail currents produced by identical pulse trains (50 pulses for A and 100 for C and E), but with increasing concentrations of deltamethrin. The concentrations of deltamethrin are indicated at the left of each set of traces. B, D, and F show tail currents produced by pulse trains of variable duration in the presence of 1 nM, 100 nM, or 5 μM deltamethrin for Na channels of wild type, *kdr* and *super-kdr*, respectively. The number of pulses in each train is indicated at the left of each set of traces. The decay phase of the largest tail currents in A and C–F was fit by the sum of one or two exponentials plus a constant. The fits are superimposed on the tail currents. The time constants (τ_1 and τ_2) for the fits were: A: $\tau_1 = 0.71$ s, $\tau_2 = 17.7$ s; C: $\tau_1 = 1.87$ s; D: $\tau_1 = 0.92$ s, $\tau_2 = 8.7$ s; E: $\tau_1 = 0.17$ s; F: $\tau_1 = 0.19$ s.

centration of deltamethrin increases, and there is a very steep concentration–response relationship.

The concentration–response relationships for all three constructs are shown in Fig. 7 D. The maximal tail current amplitude has been converted to percent modification using Eq. 2. Note that the affinity of deltamethrin is reduced almost 20-fold by the *kdr* mutation and 100-fold by the *super-kdr* double mutation. In addition, the apparent binding kinetics are reduced from 2:1 for the wild-type and kdr constructs to 1:1 for the super-kdr construct. This analysis is imperfect because the maximal amount of modification calculated with Eq. 2 is >100% for the wild-type and kdr constructs. This probably occurs because the peak conductance in ATX-II is an underestimate of $G_{Na,max}$. Furthermore, the amount of modification produced by >1 μM deltamethrin was greater than predicted by the curve fitting. This discrepancy may be due to nonspecific or second-

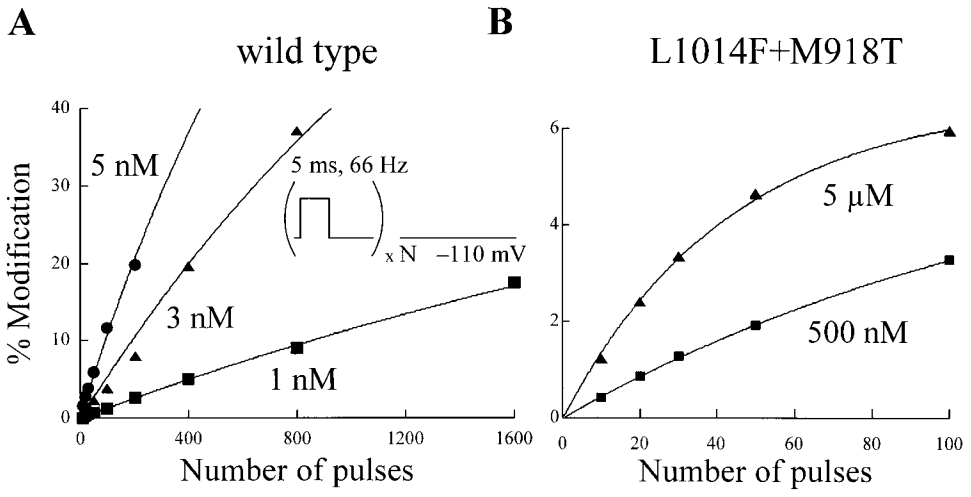

Figure 6. *Super-kdr* mutations reduce the maximal amount of modification obtainable with trains of brief depolarizations. Deltamethrin-induced tail currents were measured after trains of 5-ms depolarizations to 0 mV at 66 Hz (see inset in A for pulse protocol). The percent modification was calculated using Eq. 2 and the ATX-II factors are given in Table I. The solid curves through the data indicate the best fits to the equation: percent modification = M_{max} [1 − exp(−n/τ)], where M_{max} is the maximal percentage of modification, n is the number of pulses, and τ is an effective time constant with units of number of pulses. (A) Percent modification as a function of pulse train length for wild-type channels. ■, ▲, and ● indicate results with 1, 3, and 5 nM deltamethrin, respectively. For 1 nM, M_{max} = 48.8%, τ = 3,730 pulses. For 3 and 5 nM, the fitting assumes M_{max} = 100%; τ = 1,810 pulses for 3 nM and 873 pulses for 5 nM. (B) Modification of super-kdr channels. The ■ and ▲ indicate results with 0.5 and 5 μM deltamethrin, respectively. For 500 nM, M_{max} = 6.3% and τ = 135 pulses. For 5 μM, M_{max} = 6.7% and τ = 43.6 pulses.

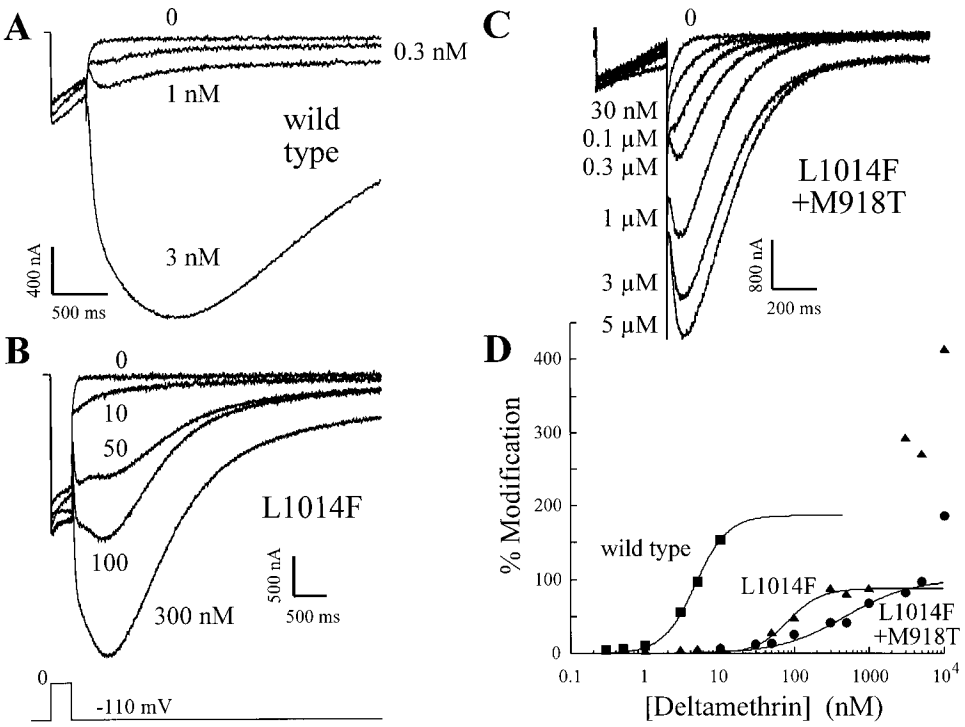

Figure 7. The *kdr* and *super-kdr* mutations reduce the potency of deltamethrin for sodium channels modified with ATX-II. All panels show Na currents measured in ≥500 nM ATX-II during a 320-ms depolarization to 0 mV and after repolarization to −110 mV. A–C show results for wild-type, *kdr*, and *super-kdr* mutants, respectively. The pulse protocol is shown below B. Note that C is shown on an expanded time scale. (D) Percent modification plotted versus deltamethrin concentration for wild-type, kdr, and super-kdr constructs (■, ▲, and ●, respectively). The fraction of modified channels was calculated using Eq. 2; the ATX-II factor was made equal to one since all experiments were conducted in maximally effective ATX-II. The solid curves through the data indicate the best fits to the equation: percent modification = $M_{max}/\{1 + (K_d/[\delta M])^n\}$, where $[\delta M]$ is the concentration of deltamethrin. Wild type (■): M_{max} = 187%, K_d = 4.71 nM, n = 2 · L1014F mutant; (▲): M_{max} = 87.6%, K_d = 82 nM, n = 2. Data for concentrations of deltamethrin >1 μM were excluded from the curve fitting; L1014F + M918T double mutant (●): M_{max} = 99.8%, K_d = 478 nM, n = 1. The datum point for 10 μM deltamethrin was excluded from the curve fitting. Each set of data was obtained with a single preparation.

ary effects of deltamethrin that occur at very high concentrations (Joy, 1994).

DISCUSSION

We find that deltamethrin effects on Para/TipE Na channels are far more potent than those previously reported for insect, vertebrate, or marine-invertebrate channels. All wild-type channels can be modified by <10 nM deltamethrin (Fig. 6). The mutations L1014F and L1014F + M918T confer 30- and 500-fold resistance to deltamethrin, respectively (Farnham et al., 1987). The *kdr* and *super-kdr* mutations reduce Na channel opening in the absence of drug by reducing the fraction of channels that open in response to depolarization (i.e, the mutations enhance closed-state inactivation). To quantify the effects of these mutations on the binding of deltamethrin, we revised a theoretical framework for quantifying the effects of pyrethroids and other Na channel activators. This analysis showed that the *kdr* and *super-kdr* mutations reduce the affinity of deltamethrin for open channels by 20- and 100-fold, respectively. In addition, these mutations reduce the time that the channel remains open once drug has bound, apparently by speeding the rate of dissociation from open channels. Thus, the mutations reduce both the potency and efficacy of insecticide action.

A Model that Describes the Mechanism of Action of Deltamethrin and of the kdr Mutations

Most of the effects of deltamethrin and the *kdr* mutations can be accounted for by the model presented in Fig. 8. The model for the effects of deltamethrin on the wild-type channel (Fig. 8 A) is very similar to that used to describe modification of vertebrate sodium channels by the alkaloids batrachotoxin and veratridine (Khodorov, 1985; Hille et al., 1987). Deltamethrin binds to open channels with much greater affinity than to rested or inactivated state channels. Insecticide-bound channels remain open as deltamethrin impedes channel closing either by inactivation or deactivation. This model accounts for most features of the time and voltage dependence of deltamethrin modification (Figs. 3 and 6 A). For simplicity, we ignore the existence of multiple closed and open states (Patlak, 1991).

The *kdr* and *super-kdr* mutations reduce sodium channel opening and thereby reduce the occupancy of the high affinity state for deltamethrin. These mutations also increase the rate of dissociation of deltamethrin from open channels (Fig. 8 B). The faster rate of dissociation is inferred because tail currents through kdr and *super-kdr* channels decay faster than those for wild type. If the rate of decay of tail current indicates the rate of dissociation of insecticide from the channel,

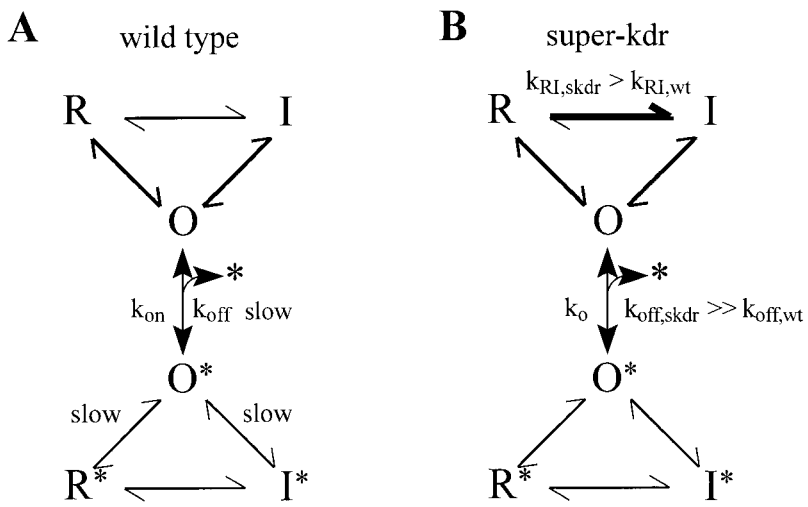

Figure 8. A model accounting for the modification of gating by deltamethrin and the *super-kdr* mutations. (A) According to voltage, the drug-free channel (top) switches between multiple states (R, resting; O, open; I, inactivated). Deltamethrin binds and unbinds only to and from the open conformation and drug binding dramatically slows transitions to closed states. The drug-bound "open" channel is conducting. For simplicity, only one rested, open, or inactivated state is shown. (B) The *super-kdr* mutations have two effects: (a) closed-state inactivation is favored so that most channels do not open before inactivating, thereby reducing the fraction of channels that enter the high affinity state for deltamethrin and reducing channel opening near threshold; and (b) the rate of dissociation of deltamethrin from open channels is faster—this accounts for the lower affinity and faster decay of insecticide-induced tail currents.

then the super-kdr mutations speed the rate of dissociation ~100-fold and this would account for the 100-fold increase in dissociation constant due to these mutations (Fig. 7). While the decay of the tail current probably represents the rate of dissociation of deltamethrin from the open channel for the super-kdr mutant, the decay of wild-type tail currents is so slow that transitions from O* → R* may be significant.

The foot-in-the-door model used to describe the mechanism of action of veratridine cannot be applied to deltamethrin without some modification. Tail currents due to modification by veratridine decay exponentially, the amplitude increases with drug concentration as expected for 1:1 binding, and the time course is independent of the amount of modification (Zong et al., 1992). In contrast, tail currents due to modification by deltamethrin become slower as the amount of modification increases and the increase in amplitude with deltamethrin concentration is very steep. For super-kdr mutants, the deltamethrin-induced tail currents have properties similar to those induced by veratridine. These results suggest that there are multiple cooperative deltamethrin binding sites per channel and that the rate of dissociation becomes slower as more than one molecule binds to a single channel. According to this interpretation, the super-kdr mutants have only one binding site per channel and cooperative binding effects are destroyed by the mutations. Although this explanation is appealing, the onset of modification by deltamethrin does not have a sigmoid time course, as often observed with higher order kinetics (Fig. 6 A). In addition, we could not describe the rate of decay of tail currents by a limited number of well defined exponential decay rates. As noted above, the dissociation of deltamethrin from wild-type channels may be so slow that transitions from O* → R* may be significant and this could account for the complex change in kinetics as modification increases. Tail currents after extensive modification by deltamethrin are hooked or have a sigmoidal onset of decay, both suggestive of transitions between multiple open states (Figs. 5 and 7). Previous studies with other sodium channels provide evidence for multiple open states (Patlak, 1991; Correa and Bezanilla, 1994) and a complete description of the decay of tail currents will likely require a more detailed state diagram than the simple three-state model that we have used.

The suggestion that there are multiple pyrethroid binding sites per channel is surprising because previous studies with pyrethroids and other sodium channel ligands gave no indication of more than one binding site. Although the α subunit of sodium channels has at least six distinct ligand binding sites, previous ligand binding studies suggest that there is only one site of each type per channel (Catterall, 1992). However, three of the ligand binding sites are for hydrophobic molecules with actions similar to those of pyrethroids: alkaloids such as batrachotoxin and veratridine bind to site 2, brevetoxins bind to site 5, and pyrethroids bind to site 6. There are strong positive allosteric interactions among these sites. For example, agonists binding to sites 5 and 6 cause a 1,000-fold enhancement of batrachotoxin binding at site 2 (Lombet et al., 1988; Trainer et al., 1993). Rather than invoke additional binding sites for pyrethroids that occur only on insect sodium channels, it is attractive to suppose that pyrethroids can occupy sites 2 and/or 5 on insect sodium channels in addition to site 6. This would provide multiple sites with cooperative binding that can account for the slowing of tail current decay that progresses with the amount of pyrethroid modification. Indeed, mutations in adjacent residues in I-S6 confer resistance to pyrethroids and batrachotoxin (Park et al., 1997; Wang and Wang, 1998; Lee et al., 1999a). Mutations in IIIS6 and IVS6 also confer resistance to pyrethroids and

batrachotoxin, respectively (Pittendrigh et al., 1997; Linford et al., 1998; Wang and Wang, 1999). Together, these mutagenesis studies suggest substantial overlap of binding sites 2 and 6. Determination of the nature and number of deltamethrin binding sites per channel will likely require isotopic ligand binding studies with insect sodium channels (Trainer et al., 1997).

Our results and the model that we propose for the mechanism of action of deltamethrin differ substantially from most previous studies with pyrethroids. Indeed, for squid axon sodium channels, the time and concentration dependence of modification by deltamethrin and other pyrethroids led to the conclusion that deltamethrin is always bound to the channels and modification occurs because the insecticide slows all gating transitions (De Weille et al., 1988). According to this scheme, pyrethroids bind readily to rested state channels and the tail current decay represents the slow deactivation of pyrethroid-bound channels, with no release of drug. It should be noted that previous studies have generally used concentrations of deltamethrin >1 μM and the time constant for decay of the deltamethrin-induced tail current was 500–3,000 ms, much faster than in our studies (Leibowitz et al., 1987; De Weille et al., 1988; Vijverberg and van den Bercken, 1990). In general, these studies did not rigorously test for preferential binding to open channels at low concentrations of deltamethrin. Instead, they used fairly high concentrations that may alter sodium channels via nonspecific or indirect mechanisms (Joy, 1994). At concentrations >1 μM, binding to closed states may indeed be significant, but such a finding does not conflict with our result that binding to open channels is far more potent.

The Insecticidal Activity of Pyrethroids

Previous electrophysiological studies of deltamethrin and related pyrethroids have produced conflicting viewpoints on the mechanism and potency of sodium channel modification and, consequently, on whether sodium channels are indeed the site of action; our results provide a means of reconciling these seemingly disparate results. First, studies of the *super-kdr* mutations provide compelling evidence that the site of action of deltamethrin is the *para* sodium channel. The L1014F and M918T double mutations confer 500-fold resistance to deltamethrin and we find correlate changes in the affinity of this drug for *para* sodium channels (Fig. 7). However, our studies also indicate that the amount of modification is exquisitely sensitive to the pattern of electrical activity. For example, Fig. 6 A shows that most channels can be modified by 1 nM deltamethrin after long trains of brief depolarizations, whereas Fig. 3 B shows no modification by 100 nM deltamethrin during a single long depolarization. Some studies that showed relatively weak effects of deltamethrin used conditions that did not elicit sodium channel opening. One of the most influential studies established two categories of pyrethroids based on their electrophysiological effects (Gammon et al., 1981). For type I pyrethroids (lacking an α-cyano group), typified by permethrin, there is a good correlation between insecticidal activity and the ability to induce electrical spiking activity in neurons after brief exposure. However, type II (α-cyano) pyrethroids, typified by deltamethrin, are disproportionately weak at inducing spiking activity. This led to the suggestion that type II pyrethroids act at sites other than insect Na channels. This study applied deltamethrin without eliciting channel opening and therefore deltamethrin binding was weak. The enhancement of pyrethroid binding by channel opening is not as great for permethrin. For permethrin at 100 nM, a single long depolarization produces substantial modification (Warmke et al., 1997), even though permethrin-induced tail currents decay more rapidly and modification by brief pulse trains is less potent than for deltamethrin (Warmke et al., 1997; our unpublished results). Thus, pyrethroids vary in the selective affinity for open channels, with type II pyrethroids like deltamethrin showing great selectivity, while type I pyrethroids such as permethrin are less selective. This variation is reminiscent of block of sodium channels by tertiary and quaternary amine local anesthetics, where the permanently charged compounds require channel opening for binding, while neutral compounds can bind to both open and inactivated states (Hille, 1977).

Common Patterns of Naturally Occurring Resistance to Insecticides

The modification of sodium channels by deltamethrin can account for the high potency of this compound as an insecticide. Once modified, sodium channels remain open after repolarization. This is lethal because, after an action potential, the cell cannot repolarize completely. This is a very efficacious mechanism because modification of a few channels is adequate to trigger spontaneous electrical activity via the Hodgkin cycle; that is, sodium channel opening increases sodium influx, which in turn depolarizes the cell and causes still more sodium channel opening. Deltamethrin augments this positive feedback loop because channel opening enhances deltamethrin modification, which in turn further stimulates channel opening. The *kdr* and *super-kdr* mutations, which occur naturally and so represent changes that preserve viability in the field, defeat this toxicity by a combination of effects. First, 70–80% of the sodium channels never open due to enhanced closed-state inactivation. In well-studied cases of axonal conduction, there are "extra" sodium channels that provide a substantial safety factor for rapid conduction (Hodgkin, 1975). Reducing sodium channel opening

by enhancing closed state inactivation reduces the occupancy of the high affinity state for deltamethrin and reduces the number of channels that can support electrical spiking. Closed-state inactivation is of greatest importance near the threshold for action potential generation; the speeding of this process by the *kdr* and *super-kdr* mutations will also serve to inhibit spontaneous electrical spiking activity. The *kdr* and *super-kdr* mutations also reduce the potency and efficacy of deltamethrin. This is seen most dramatically after trains of brief depolarizations (Figs. 5 and 6); for super-kdr channels, modification does not continuously accumulate during the pulse train, so only a small fraction of channels are modified even with very high concentrations of deltamethrin.

The effect of *super-kdr* mutations on Na channels is analogous to the effect of the resistance to dieldrin (*Rdl*) mutation on γ-aminobutyric acid–gated channels (Zhang et al., 1994). In both cases, identical point mutations are repeatedly isolated in the field, indicating that the mutation is extraordinarily effective at conferring resistance while preserving viability. Both the *Rdl* and *super-kdr* mutations confer resistance by a dual mechanism: they reduce the affinity of the insecticide for its receptor and modify channel gating so as to offset the effect of insecticide. The *Rdl* mutation reduces the affinity of cyclodienes for channel block and also slows channel desensitization so that unblocked channels remain open longer and compensate for blocked channels. Likewise, the *super-kdr* mutations reduce the affinity of insecticide, but, in contrast to the *Rdl* mutation, channel opening is reduced to compensate for ligand-induced opening.

Finally, our study indicates a possible means of overcoming pyrethroid resistance in the field. Fig. 1 indicates that the effect of the *super-kdr* mutation on Na channel opening can be overcome with an agent that acts like ATX-II because this toxin apparently inhibits closed-state inactivation. A comparison of Fig. 6 B with 7 indicates that the ATX-II–induced slowing of inactivation can result in increased modification of super-kdr channels by deltamethrin. Thus, small molecules that mimic the action of ATX-II might be combined with pyrethroids to sensitize pyrethoid-resistant insects. Small molecules mimics of ATX-II are known (Wang et al., 1990).

This work was supported by grants to P.N.R. Usherwood (42/INS2999, 42/S10233) and A.L. Devonshire (42/INS2999) from the British Biotechnology and Biological Sciences Research Council.

Submitted: 3 September 1999
Revised: 24 January 2000
Accepted: 24 January 2000
Released online: 28 February 2000

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