Mutations of the *para* Sodium Channel of *Drosophila melanogaster* Identify Putative Binding Sites for Pyrethroids


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ABSTRACT

The effects of two pyrethroids on recombinant wild-type and mutant (pyrethroid-resistant) Na⁺ channels of *Drosophila melanogaster* have been studied. Three mutations that confer resistance (*kdr/superkdr*) to pyrethroids were inserted, either individually or in combination, into the *para* Na⁺ channel of *D. melanogaster*: L1014F in domain IIS6, M918T in the IIS4-S5 linker, and T929I in domain IIS. Channels were expressed in *Xenopus laevis* oocytes and the effects of the pyrethroids permethrin (type I) and deltamethrin (type II) on Na⁺ currents were investigated using voltage clamp. The Na⁺ channels deactivated slowly after deltamethrin treatment, the resultant “tail” currents being used to quantify the effects of this pyrethroid. The Hill slope of 2 for deltamethrin action on the wild-type channel and the mutant L1014F channel is indicative of cooperative binding at two or more sites on these channels. In contrast, binding to the mutants M918T and T929I is noncooperative. Tail currents for the wild-type channel and L1014F channel decayed biphasically, whereas those for M918T and T929I mutants decayed monophasically. The L1014F mutant was 20-fold less sensitive than the wild-type to deltamethrin. Surprisingly, the sensitivity of the double mutant M918T+L1014F to deltamethrin was similar to that of M918T alone, whereas the sensitivity of T929I+L1014F was >30,000-fold lower than that of T929I. Permethrin was less potent than deltamethrin, and its binding to all channel types was noncooperative. The decays of permethrin-induced tail currents were exclusively monophasic. These findings are discussed in terms of the properties and possible locations of pyrethroid binding sites on the *D. melanogaster* Na⁺ channel.

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ABBREVIATIONS: ATX, anemone (*Anemone sulcata*) toxin.
methrin treatment, tail currents of the L1014F mutant decay faster than those of the wild-type channel, but they remain biphasic with time constants ($\tau$) dependent on the deltamethrin concentration (Vais et al., 1998, 2000a). In contrast, the tail current of the double mutant L1014F+M918T is best fitted by a single exponential function with a $\tau$ that is independent of the deltamethrin concentration (Vais et al., 2000a). The L1014F mutant is 20-fold less sensitive to deltamethrin than the wild-type channel, but both channels have Hill coefficients of 2 (Vais et al., 2000a). Although the number of binding sites is not defined by the Hill coefficient, the coefficient does set a lower limit; so it may be concluded that there are at least two such sites for deltamethrin on wild-type and L1014F channels and that these interact in a positively cooperative manner. In contrast, the Hill coefficient for deltamethrin action on M918T+L1014F is 1 (Vais et al., 2000a), suggesting that, together, the mutations L1014F and M918T reduce cooperativity and/or the number of binding sites for deltamethrin, but we do not know whether this is due solely to the presence of the M918T mutation. Therefore, we have compared the effects of deltamethrin on the single mutant M918T with its effects on the double mutant M918T+L1014F. We have also investigated the effects of another superkdr mutation, T292I, found in the diamondback moth (Schuler et al., 1998), expressed either alone or in combination with L1014F. The residue Thr292 is located in II5 and is equivalent to Thr885 in the Na$^+$ channel of human skeletal muscle. Mutation of Thr885 to T885M (one of the mutations responsible for hyperkalaemic periodic paralysis) shifts Na$^+$ channel activation in a hyperpolarizing direction and dramatically impairs slow inactivation (Cummins and Sigworth, 1996).

It is possible that further insight into the binding sites for pyrethroids may be obtained by comparing the actions of type I and type II pyrethroids. Herein, we present quantitative data on the effects of permethrin on wild-type and mutant (L1014F, M918T, T929I, M918T+L1014F, and T929I+L1014F) channels that lend support to this possibility. We show that permethrin and deltamethrin preferentially stabilize the open conformation of the recombinant Na$^+$ channel of D. melanogaster and that this action is impaired by the M918T and T929I mutations. Possible binding sites for pyrethroids are proposed and a mechanism is suggested to account for the actions of permethrin and deltamethrin on the wild-type channel and the influences of the resistance mutations on the actions of these insecticides.

**Materials and Methods**

The $\text{para}$ Na$^+$ channel cDNA construct ($\text{para}$ 13-5) was kindly provided by Dr. J. Warnke (Merck, Rahway, NJ) (Warnke et al., 1997). The TipE construct (pGHitpE) was used previously by Vais et al. (2000a). Point mutations (L1014F, M918T, and T929I) were introduced into the $\text{para}$ cDNA using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). $\text{Para}$ and TipE RNA transscripts were synthesized from Not-1 linearized plasmid templates using the T7 mMESSAGE mACHINE kit (Ambion, Austin, TX), according to the supplier’s recommended protocols.

$\text{X. laevis}$ oocytes were isolated and injected according to standard procedures. A cRNA transcript (1 ng/ml) of either the wild-type channel or a mutant channel was mixed with TipE (Feng et al., 1995) and RNAase-free water, the final mixture having a ratio by volume of 1 part $\text{para}$/1 part TipE/3 parts sterile H$_2$O. Oocytes were injected with 50 nl of the transcript solution and incubated at 19°C for 2 to 5 days in ND-96 GPT solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl$_2$, 1.8 mM CaCl$_2$, 2.5 mM sodium-pyruvate, 0.5 mM theophylline, 50 mg/ml gentamicin sulfate, and 5 mM HEPES, pH 7.5) before recording.

Two-electrode voltage-clamp experiments were performed using a CA-1 amplifier (Dagan, Minneapolis, MN). The bath solution was ND-96 (but without sodium-pyruvate, theophylline, and gentamicin sulfate). In some experiments, the Na$^+$ concentration of ND-96 was reduced by equimolar replacement of NaCl with $\text{N}$-methyl-$\beta$-glucamine, to achieve better voltage control in experiments on oocytes expressing large Na$^+$ currents or when using the sea anemone (Anemonia sulcata) toxin ATX-II to inhibit Na$^+$ channel inactivation. ATX-II is not a competitor of pyrethroids at Na$^+$ channels, but uncouples activation from inactivation of a Na$^+$ channel by binding to a site on the extracellular end of segment IVS4; i.e., ATX-II binding seemingly allows movements of IVS4 required for activation, but not those for fast inactivation (Rogers et al., 1996). Experiments were performed at room temperature (21–23°C). Agar bridges with platinum wires of resistance $<7$ kΩ and voltage-messuring glass micropipettes with resistances $\leq$1 MO when filled with 1 M KCl were used to improve the frequency response of the voltage clamp. The latter was additionally improved by using low-resistance, current-injection glass micropipettes ($\approx$0.5 MΩ when filled with 0.7 M KCl + 1.7 M potassium-citrate). Data were acquired using the HEKA Pulse Program (Digitimer Ltd., Welwyn Garden City, Hertfordshire, UK), and most analyses were performed with its companion program PulseFit. Linear leak and capacitive currents were subtracted with P/5 steps from $\approx$120 mV. Data were sampled at 50 kHz and filtered at 10 kHz unless indicated otherwise.

Technical grade or purer ($\text{oS}$-deltamethrin and the [1R-cis] isomers of permethrin were kindly provided by Dr. B. Khambay (Rothamsted Research, Harpenden, Hertfordshire, UK). Stock solutions (10$^{-2}$ M) of the two pyrethroids were prepared in ethanol. After their dilution with ND-96, the final concentration of ethanol in the oocyte-bathing saline did not exceed 0.1%. Control experiments undertook to check the effect of 0.1% ethanol on the sodium channels gave negative results. ATX-II was purchased from Calbiochem (La Jolla, CA). All other chemicals were from Sigma Chemical (Poole, Dorset, UK).

**Results**

The Single Mutations M918T and T292I Promote Closed-State Inactivation. Deltamethrin slows the open-inactivated-closed (deactivated)–state transitions of voltage-activated Na$^+$ channels in a use-dependent manner (Vais et al., 2000a). Therefore, we first determined the effects of the mutations M918T and T292I, either alone or in combination with L1014F, on channel opening. The single mutation M918T slightly (>2 mV), but significantly ($P < 0.001$), shifted the half-maximal potential ($V_{1/2}$) for activation in a depolarizing direction (Table 1). Steady-state inactivation, measured using conditioning pulses of 200 ms, was unaffected by this mutation, although the inactivation kinetics was significantly faster than for the wild-type channel (Table 1; Vais et al., 2001). The mutation T292I had no effect on channel activation, but significantly ($P < 0.0001$) shifted the midpoint potential ($V_{1/2}$) for steady-state inactivation by $\approx$5 mV in the hyperpolarizing direction (Fig. 1; Table 1). The onset rate of inactivation of T292I was also increased, especially around $\approx$40 mV, the threshold for Na$^+$ channel activation (Warnke et al., 1997) (Table 1; Fig. 1). The voltage dependencies of activation and steady-state inactivation and the rates of onset of inactivation of channels with the double mutations M918T+L1014F and T292I+L1014F were similar to those for the wild-type channel (Table 1). In contrast, the
rates of recovery from inactivation of channels with these double mutations were similar to those of the corresponding single mutants (Fig. 1).

By comparing maximal conductances ($G_{\text{max}}$) before and after application of ATX-II, it was possible to determine the effect of the resistance mutations on the level of closed state inactivation (Patlak, 1991; Warmke et al., 1997; Vais et al., 2000a). ATX-II increased $G_{\text{max}}$ of oocytes expressing either the M918T mutant channel or the L929I mutant channel by ~500%, which indicates that a large fraction of these channels normally (i.e., in the absence of ATX-II) inactivate without opening. In oocytes expressing the wild-type channel, a smaller fraction of the channels inactivate without opening because ATX-II increased $G_{\text{max}}$ by only 200%. It is noteworthy that similar increases in $G_{\text{max}}$ to those obtained with the single mutants were obtained with L1014F, M918T+L1014F, and T929I+L1014F.

Deltamethrin-Induced Tail Currents for the Single Mutants M918T and T929I. We have shown previously that deltamethrin preferentially interacts with the open state of the D. melanogaster wild-type Na$^+$ channel (Vais et al., 1998, 2000a). Thus, in principle, the action of this pyrethroid could be examined using either pulse protocols that promote channel opening by enhancing recovery from inactivation or by using ATX-II to inhibit inactivation from both closed and open states (Warmke et al., 1997). Although clear differences in the sensitivities of the wild-type and mutant channels to deltamethrin emerged using pulse protocols, these protocols could not be used to estimate the relative affinities of deltamethrin for the wild-type and mutant channels. This is due, in part, to pronounced closed state inactivation of the mutant channels (Pauron et al., 1989; Vais et al., 1998). It was relatively easy to construct a pulse train that induced 100% modification of wild-type channels by 1 to 10 nM deltamethrin, because this pyrethroid induces tail currents that decay very slowly (Vais et al., 2000a). In contrast, the deltamethrin-induced tail currents for the mutants M918T and T929I decay very rapidly. As a result, the macroscopic open-state lifetimes and the time constants for recovery from inactivation of these channels prevented us from designing pulse train frequencies giving 100% modification. In fact, it was not possible to modify more than ~80% of T929I channels and ~5% of M918T channels using pulse trains, even when the concentration of deltamethrin was raised to 10 μM (the maximum usable concentration because of solubility constraints) (Fig. 2A). Nevertheless, the tail current amplitudes were increased when the pulse frequency was raised (Fig. 2B), which supports our view that deltamethrin preferentially targets the open state of the D. melanogaster Na$^+$ channel. This phenomenon cannot be caused by accumulation of slowly activating, pyrethroid-modified channels (Tabarean and Narahashi, 2001), because tail current amplitude was also increased when an increase in pulse train frequency was accompanied by a reduction in pulse train duration (Fig. 2B). In the studies reported herein, we have inhibited closed- and open-state inactivation with ATX-II (500 nM) so that equivalent numbers of deltamethrin-modified wild-type, M918T, and T929I channels were obtained after a single long (320-ms) step depolarization (to 0 mV). The decays of pyrethroid-induced tail currents are not affected by ATX-II (e.g., Fig. 2C, for the M918T mutant).

The sensitivity of the T929I mutant to deltamethrin was higher than that of the M918T mutant. Figure 2D illustrates a family of tail currents for T929I obtained with increasing concentrations of deltamethrin. The decay of the T929I tail current was preceded by a “hook” current. Hook currents were also obtained with the wild-type channel and L1014F mutant (Fig. 2, E and F) and, to a lesser extent, with the M918T mutant (Fig. 2B). The amplitude of the hook current was dependent on the concentration of pyrethroid. Despite the presence of a hook current, the tail current decayed monophonically with a time constant ($\tau$) of $0.96 \pm 0.02$ s ($n = 6$) that was independent of the concentration of deltamethrin. In contrast, tail currents for the wild-type and L1014F mutant decayed biphasically, with time constants that were dependent on deltamethrin concentration (Fig. 2, E and F, Table 2).

Concentration-Response Relationships for Deltamethrin Action on the M918T and T929I Mutants. Data presented in Fig. 3 show that the affinity of deltamethrin for the M918T mutant was ~200 times less than its affinity for the wild-type channel: the apparent dissociation constants ($K_d$) were 995 ± 29 nM (S.E. of the fit) for the mutant channel and 4.7 nM for the wild-type channel. The Hill coefficients were $n_H = 2$ (chosen by iteration as a fixed constant during curve fitting to the concentration-response relationship; goodness of fit was 0.999; Fig. 3) for the wild-type channel and $n_H = 0.8 \pm 0.1$ for the M918T channel. The T929I mutant had a $K_d$ of 45 ± 1.1 nM (Fig. 3) and a Hill coefficient $n_H = 0.8 \pm 0.1$ (Table 2). Significantly, the $K_d$ values for T929I and M918T are ~10 and ~200 times greater, respectively, than that for the wild-type channel.

The Double Mutants T929I+L1014F and M918T+L1014F. The double mutant T929I+L1014F ($K_d = 1.5 \pm 1.1$ nM; Hill coefficient $n_H = 0.3 \pm 0.04$; Fig. 4B) was ~300,000 times less sensitive to deltamethrin than the wild-type channel and ~30,000 times less sensitive than the single mutant channel.

### TABLE 1

<table>
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<th>Wild-Type</th>
<th>L1014F</th>
<th>M918T</th>
<th>T929I</th>
<th>M918T+L1014F</th>
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<td>$V_{1/2}$ (mV)</td>
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<td>$-14.2 \pm 2.4^{**}$</td>
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<td>$6.78 &lt; 0.3$</td>
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<td>$V_{1/2}$ (mV)</td>
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<td>$-38.2 \pm 0.6^{***}$</td>
<td>$-42.2 &lt; 1.4$</td>
<td>$-48.5 \pm 0.8^{***}$</td>
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<td>$k$ (mV)</td>
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<td>$1.34 &lt; 0.1$</td>
<td>$0.95 &lt; 0.1^{***}$</td>
<td>$1.45 &lt; 0.1^{***}$</td>
<td>$1.33 &lt; 0.1$</td>
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<td>$8.7 &lt; 1.0^{***}$</td>
<td>$6.6 &lt; 0.9^{***}$</td>
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* * $P < 0.01$; *** $P < 0.001$ relative to corresponding values for the wild-type channel.
T929I (Fig. 4). In contrast, the sensitivity of the double mutant M918T+L1014F to deltamethrin was similar to that of the single mutant channel M918T: the $K_d$ values (in nanomolar units) were $479 \pm 10$ and $995 \pm 29$, respectively (Fig. 4; Table 2). The tail current decay time constants for the two double mutants were independent of deltamethrin concentration ($\tau = 0.55 \pm 0.01$ s ($n = 4$) for M918T+L1014F and $\tau = 1.16 \pm 0.02$ s ($n = 6$) for T929I+L1014F).

**Interaction of Permethrin with Wild-Type and Mutant Channels.** When permethrin was applied to oocytes expressing the D. melanogaster wild-type Na$^+$ channel, tail currents with monoeXponential decays were elicited. Like deltamethrin, permethrin preferentially binds to the open state of the wild-type channel; i.e., its effect was greater after a pulse train than after a single long depolarization (Fig. 5A).

The effect of permethrin was enhanced either by increasing the number of pulses in the train (Fig. 5B) or by increasing pulse amplitude (Fig. 5C). The tail current decay time constant ($\tau = 746 \pm 18$ ms; $n = 6$) was independent of permethrin concentration. The concentration-response relationship for permethrin, obtained using 500 nM ATX-II to inhibit the L1014F mutant, was $1.01 \pm 0.01$ s ($n = 4$) for M918T+L1014F and $1.11 \pm 0.02$ s ($n = 6$) for T929I+L1014F.

**Effects of Permethrin on the L1014F Mutant.** The $K_d$ value for permethrin action on the L1014F mutant was $48.3 \pm 1.2$ nM and the Hill coefficient was $n_H = 0.98 \pm 0.16$.

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**Fig. 1.** Onset of and recovery from inactivation are influenced by the T929I mutation. The onset of inactivation of the T929I mutant Na$^+$ channel (■) is faster than that of the para wild-type Na$^+$ channel (○) around the threshold for channel activation (~40 mV). In contrast, the onset rate of inactivation of the double mutant T929I+L1014F (●) is closer to that of the wild-type channel. Onset of and recovery from inactivation were studied by applying a conditioning pulse (0.5–256-ms duration) at each membrane potential indicated on the abscissa, before a 10-ms test pulse to ~10 mV. For recovery from inactivation, the conditioning pulse was preceded by a 10-ms depolarizing (activation) step to ~10 mV. In both the onset and the recovery experiments, a 3-s delay preceded each pulse set. Onset and recovery time constants for each membrane potential on the abscissa were determined by fitting single exponentials to the decays (for onset of inactivation) or rises (for recovery from inactivation) of plots of peak inward Na$^+$ current obtained during the test pulses versus conditioning pulse duration. Each datum point represents the mean of at least five experiments, and the error bars are S.E.M. Similar data (not shown) were obtained for the M918T mutant and the M918T+L1014F mutant.

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**Fig. 2.** The single mutants M918T and T929I exhibit different sensitivities to deltamethrin. A, percentage of channels modified by deltamethrin is raised by increasing the number ($n$) of 5-ms pulses (from ~100 to 0 mV and back to ~100 mV) delivered in trains at 66 Hz. With 10 µM deltamethrin (maximum concentration used), a maximum modification of 5% of M918T channels (●) and 80% of T929I channels (▲) was obtained. The percentage of modified channels was estimated according to Vais et al. (2000a). Data points were fitted with the exponential equation $M = M_{\text{max}} [1 - \exp(-n n_e)]$ (solid curves), where $M$ is the percentage of modified channels and $n_e$ is a constant indicating the number of pulses required to induce (1–1/e) modification from the maximum ($M_{\text{max}}$), as a result of the action of the pyrethroid. The fitting parameters for M918T were $M_{\text{max}} = 5.0 \pm 0.2$% (S.D., $n = 4$) and $n_e = 46 \pm 4$% (S.D., $n = 4$). The fitting parameters for T929I were $M_{\text{max}} = 80.8 \pm 1.2$% (S.D., $n = 3$) and $n_e = 96 \pm 4.3$% (S.D., $n = 3$), B, for the M918T mutant, higher magnitude tail currents (recorded at ~110 mV) were induced by 10 µM deltamethrin when applying 100 pulses of 3-ms duration (at 0 mV) at 111 Hz (trace 1) than when applying 100 pulses of 5 ms (at 0 mV) at 66 Hz (trace 2). For both sets of experiments, the pulse trains were delivered from a holding potential of ~100 mV, a voltage at which the time constant for recovery from inactivation is ~1 ms. C, tail currents induced by 10 µM deltamethrin, recorded from two oocytes expressing similar numbers of M918T channels [$G_{\text{max}} = 55.3 \mu S$ (trace 1) and 34.7 µS (trace 2)]. The oocytes were subjected to either 100 conditioning pulses of 5-ms (at 0 mV) duration at 66 Hz (trace 1) or, in oocytes exposed to 500 nM ATX-II, to a single 320-ms pulse (to 0 mV) (trace 2). In both cases, conditioning pulses were delivered from a holding potential of ~100 mV, and tail currents were recorded at ~110 mV. D, a family of tail currents recorded from an oocyte expressing the T929I mutant and exposed to increasing concentrations (nanomolar) of deltamethrin (indicated below each trace). The oocyte was pretreated with 500 nM ATX-II and the pulse protocol (presented below traces) consisted of a single 320-ms pulse (to 0 mV), from a holding potential of ~100 mV, followed by a 12-s repolarization to ~110 mV. This protocol (ATX-II protocol) was used on all six constructs. E, tail current recorded from an oocyte expressing the wild-type channel ($G_{\text{max}} = 163$ µS) and exposed to 1 nM deltamethrin (ATX-II protocol). The tail current decay was best fitted with two exponentials, with time constants $\tau_1 = 1.01$ s and $\tau_2 = 19.2$ s. F, tail current recorded from an oocyte expressing the L1014F mutant (ATX-II protocol). The tail current decay was best fitted with two exponentials, with time constants $\tau_1 = 1.2$ s and $\tau_2 = 7.5$ s. The dotted lines represent the holding currents required to clamp the oocyte at ~100 mV.
(S.E. of the fit; n = 3). The 4-fold increase in $K_d$ compared with the wild-type channel was accompanied by a faster decaying tail current, but one that could still be fitted by a single exponential function ($\tau = 233 \pm 8$ ms; S.E.M., n = 4) (Table 2). Therefore, the L1014F mutation reduced the efficacy and the potency of permethrin in slowing channel deactivation.

**Sensitivity of the M918T Mutant to Permethrin.** The M918T mutation almost abolished sensitivity to permethrin, although rapidly decaying tail currents ($\tau = 96.8 \pm 3.4$ ms; S.E.M., n = 3) were observed at high (>1 $\mu$M) permethrin concentrations (Fig. 6A). Because of its low sensitivity to permethrin and the limited solubility of the pyrethroid, only the “foot” of a concentration-response relationship for the mutant M918T could be obtained (Fig. 6B, □).

**Sensitivity of the T929I Mutant to Permethrin.** Figure 7A shows that permethrin-induced tail currents for the T929I mutant decayed faster (~8 times; Table 2) than their wild-type counterparts. The concentration-response relationship for permethrin action on T929I that is illustrated in Fig. 7B (■) shows a 10-fold decrease in sensitivity to permethrin ($K_d = 150.4 \pm 9.9$ nM; S.E. of the fit, n = 4) compared with the wild-type channel. This reduction in sensitivity was quantitatively similar to that seen with deltamethrin.

**Sensitivity of the Double Mutants M918T+L1014F and T929I+L1014F to Permethrin.** The double mutant M918T+L1014F was slightly more sensitive to permethrin, as it was to deltamethrin, than the single mutant M918T. This was the case irrespective of whether a pulse train or ATX-II was used to promote the action of the pyrethroid. Slowing of inactivation by the L1014F mutation (values for $\tau_{\text{decay}}$ at $-10$ mV for M918T and M918T+L1014F are presented in Table 1) would have reduced the efficacy of the conditioning pulse train in our pulse train experiments, which may have accounted for part of the higher sensitivity of the double mutant to permethrin. However, the results of the ATX-II experiments clearly show that the affinity of the double mutant for permethrin was higher than that of the M918T channel (Fig. 6B). Unfortunately, the low solubility of pyrethroids in aqueous solutions, coupled with the very low sensitivity of the mutants to permethrin, precluded us from quantifying differences in their affinities for this pyrethroid. The sensitivity of the double mutant T929I+L1014F to permethrin was so low (Fig. 7B, ▼; Table 2) that it was not possible accurately to obtain an estimate for $K_d$. Despite this limitation, we are able safely to conclude that permethrin and deltamethrin have qualitatively similar effects on the two double mutants, M918T+L1014F and T929I+L1014F.

**Discussion**

Mutations M918T and T929I of the para Na$^+$ channel of *D. melanogaster* induce closed state inactivation. This could explain why the M918T mutant seemed to express very weakly in *X. laevis* oocytes (Lee et al., 1999a), a finding that has led to the speculation that effective expression in vivo of a superkdr mutation may only arise in the presence of a kdr mutation (Lee et al., 2000). Although our data in Fig. 1 seemingly lend support to this speculation, we have also demonstrated that good functional expression of the M918T mutant can be obtained in the absence of a kdr mutation and that many of the actions of a pyrethroid on the para Na$^+$ channel of *D. melanogaster* are best studied when closed state inactivation is inhibited by ATX-II.

We have provided evidence that both permethrin and deltamethrin preferentially bind to the open conformation of the *D. melanogaster* Na$^+$ channel to stabilize the open state, thereby slowing inactivation and deactivation. This results in a sustained inward current during a step depolarization (Narahashi, 1996) and a prolonged tail current after repolarization, the latter having been used quantitatively to measure pyrethroid action (Tatebayashi and Narahashi, 1994; Vais et al., 1998, 2000a). The concentration-response relationship for permethrin action on the wild-type *D. melanogaster* Na$^+$ channel has a Hill coefficient of 1, which suggests that this pyrethroid has either a single binding site or multiple, but independent, binding sites. In contrast, the concentration-response relationship for deltamethrin has a Hill coefficient of 2, suggesting that it binds to at least two sites. It seems that both deltamethrin and permethrin bind to multiple sites, but that the binding of deltamethrin is cooperative, whereas that of permethrin is not. Clearly, a detailed structure-activity study is required to ascertain the relationship between cooperative binding and pyrethroid structure. Significantly, the superkdr mutations M918T and T929I (in either the presence or absence of L1014F) are the only mutations identified so far that affect the Hill coefficient for deltamethrin (Vais et al., 2001; Tan et al., 2002). It is also interesting to note that the effects of deltamethrin and permethrin on channels with these mutations are qualitatively similar.

It is generally accepted that pyrethroids bind to insect Na$^+$

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**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>Wild-Type</th>
<th>L1014F</th>
<th>M918T</th>
<th>T929I</th>
<th>M918T+L1014F</th>
<th>T929I+L1014F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deltamethrin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ (nM$^c$)</td>
<td>$4.7 \pm 0.1$</td>
<td>$90 \pm 2.1$</td>
<td>$995 \pm 29$</td>
<td>$45 \pm 1.1$</td>
<td>$479 \pm 10$</td>
<td>$1.5 \pm 1.1$ M$^a$</td>
</tr>
<tr>
<td>$n_H$ $^d$</td>
<td>$2^e$</td>
<td>$1.8 \pm 0.14$</td>
<td>$0.8 \pm 0.1$</td>
<td>$0.8 \pm 0.1$</td>
<td>$0.9 \pm 0.03$</td>
<td>$0.3 \pm 0.04$</td>
</tr>
<tr>
<td>Tail decay (s)</td>
<td>$\tau_1 = 1.9 \pm 0.3^d$</td>
<td>$\tau_1 = 1.9 \pm 0.3^d$</td>
<td>$0.61 \pm 0.03$</td>
<td>$0.96 \pm 0.02$</td>
<td>$0.55 \pm 0.01$</td>
<td>$1.16 \pm 0.02$</td>
</tr>
<tr>
<td></td>
<td>$\tau_2 = 215 \pm 63^d$</td>
<td>$\tau_2 = 7.4 \pm 0.7^d$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permethrin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_d$ (nM$^c$)</td>
<td>$11.9 \pm 0.8$</td>
<td>$48.3 \pm 1.2$</td>
<td>$--^b$</td>
<td>$150.4 \pm 9.9$</td>
<td>$--^b$</td>
<td>$2.3 \pm 9.5$ M$^a$</td>
</tr>
<tr>
<td>$n_H$ $^d$</td>
<td>$1.03 \pm 0.07$</td>
<td>$0.98 \pm 0.16$</td>
<td>$0.9 \pm 0.05$</td>
<td>$0.29 \pm 0.06$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tail decay (ms)</td>
<td>$\tau_1 = 746 \pm 18$</td>
<td>$233 \pm 8$</td>
<td>$96.8 \pm 3.4$</td>
<td>$91.1 \pm 3.2$</td>
<td>$86.6 \pm 3$</td>
<td>$114.3 \pm 12.7$</td>
</tr>
</tbody>
</table>

$^a$ Valid for all $K_d$ values given in this table, except those in the last column (data for the T929I+L1014F mutant).

$^b$ It was not possible accurately to determine $K_d$ because of the poor solubility of the pyrethroids in aqueous solution.

$^c$ Because of the damaging effects of high concentrations of deltamethrin on oocytes expressing the wild-type channel, it was not possible to obtain a plateau for the concentration-response relationship. As a result, the relationship was fitted by iteration with a fixed value for the Hill coefficient $n_H$.

$^d$ Values determined at 5 nM deltamethrin.

$^e$ Values determined at 1 $\mu$M deltamethrin.
channels at sites that are different from those for other Na⁺ channel modulators (Narahashi, 1998). Do permethrin and deltamethrin bind to the para Na⁺ channel of D. melanogaster at sites Leu1014, Met918, and Thr929? Without definitive data from ligand binding studies, it is not possible to give an unequivocal answer to this question. The mutation L1014F reduces the affinities of permethrin and deltamethrin in quantitatively similar manner. The leucine residue at position 1014 lies in the middle of IIS6. However, in some pyrethroid-resistant insects, Kdr-like mutations are located at equivalent positions in other domains [e.g., domain I (e.g., Lee et al., 1999)]. Possibly pyrethroids bind to all such Kdr sites on insect wild-type Na⁺ channels (Wang et al., 2001). The location of Leu1014 in the membrane phase might make it more accessible to lipophilic pyrethroids such as permethrin and deltamethrin. However, the possibility remains that the kdr sites are not pyrethroid binding sites, but that kdr mutations induce conformational changes in the Na⁺ channel protein that restrict the binding of pyrethroids to other sites on the channel (e.g., the IIS4-S5 linker). It is important to note that the resistance induced by a kdr mutation, such as L1014F, is not very large and could conceivably result from a small reduction in channel open time.

Are the positions of residues Met918 and Thr929 on the IIS4-S5 linker consistent with the effects of pyrethroids on insect Na⁺ channel gating, in terms of slowing activation (Tabarean and Narahashi, 2001), inactivation (Narahashi, 1996), and deactivation (Tatebayashi and Narahashi, 1994; Vais et al., 1998, 2000a)? The IIS4-S5 linker is a cytoplasmic domain that may form part of the docking site for the fast inactivation particle (Holmgren et al., 1996; McPhee et al., 1998) [i.e., the IFM amino acid sequence from the cytoplasmic loop between domains III and IV (Catterall, 2000)]. The IIS4-S5 linker is located near the intracellular mouth of the channel pore and is connected to the IIS4, one of the four S4 segments that serve as voltage sensors for channel gating (Benzanilla, 2000). The possible role of the S4-S5 linker of domain IV in fast inactivation has been demonstrated in the mutagenesis studies of McPhee et al. (1998). By binding to residues Met918 and Thr929 on the IIS4-S5 linker, pyrethroids could impair fast inactivation by inhibiting binding of the fast inactivation particle without drastically altering

![Figure 3](image1.png)

**Fig. 3.** Concentration-response relationships for deltamethrin action on M918T (▲) and T929I (■) mutant Na⁺ channels: data obtained from oocytes subjected to the ATX-II protocol. Each datum point represents the mean of at least three experiments and the error bars are S.E.M. Each set of data was fitted with a Hill equation (solid line); the fitting parameters are given in Table 2. For comparison, the concentration-response relationship for deltamethrin action on the wild-type channel (○) is also included. As stated previously (Vais et al., 2000a), values >100% for the percentage of channels (M%) modified by deltamethrin occur because the maximal conductance obtained after ATX-II treatment underestimates the number of Na⁺ channels expressed in an oocyte and because Na⁺ channel open probability is increased by the pyrethroid (Narahashi, 1998).

![Figure 4](image2.png)

**Fig. 4.** Introduction of the L1014F mutation into the T929I mutant (to give T929I+L1014F) reduces sensitivity to deltamethrin by ~30,000-fold. In contrast, introduction of the L1014F mutation into the M918T mutant (to give M918T+L1014F) has no effect on sensitivity to deltamethrin. A, comparison of tail currents recorded (at ~110 mV) from oocytes expressing similar numbers of Na⁺ channels (G∞ = 86.6 μS) for T929I and 100 μS for (T929I+L1014F). B, concentration-response relationships for deltamethrin action on the single mutant T929I (■) and the double mutant T929I+L1014F (○), compared with those for the single mutant M918T (▲) and the double mutant M918T+L1014F (△), obtained from oocytes treated with ATX-II. Each datum point represents the mean of at least three experiments and the error bars are S.E.M. Each set of data was fitted with a Hill equation (solid line); the fitting parameters are given in Table 2. Values >100% for the percentage of channels (M%) modified by deltamethrin occur because the maximal conductance obtained after ATX-II treatment underestimates the number of Na⁺ channels expressed in an oocyte and because Na⁺ channel open probability is increased by the pyrethroid (Narahashi, 1998).
other biophysical properties of the channel. However, when fast inactivation is inhibited by ATX-II, the action of permethrin and deltamethrin is largely unaffected, which supports the contention that the effect of these compounds on deactivation is also important.

How could binding of pyrethroid to the S4-S5 linker of domain II influence channel deactivation? Voltage-dependent activation of a Na⁺ channel is believed to arise through the outward movement of the voltage-sensing S4 segments of its four domains during membrane depolarization (Catterall, 1986). The return of these segments to their original positions after repolarization leads to channel closure (deactivation), which can be most readily observed when fast inactivation is inhibited by ATX-II. According to Horn (2000), the transmembrane outward movement of a IIS4 segment during activation may be substantial, which, if correct, could pull the N-terminal residues of the IIS4-S5 linker closer to, or

Fig. 5. Permethrin preferentially affects the open state of the para Na⁺ channel of Drosophila. A, comparison of tail currents recorded (at −110 mV) from an oocyte expressing the para wild-type Na⁺ channel after application of 10 nM permethrin. Tail currents were elicited after depolarizing the oocyte to 0 mV either with a 500-ms pulse (trace 1) or with a train of 100 pulses of 5 ms, each interrupted by a 10-ms repolarization to −100 mV to allow channels to recover from inactivation (trace 2). B, family of tail currents, elicited by 10 nM permethrin, recorded (at −110 mV) from an oocyte expressing the para wild-type Na⁺ channel. The oocyte was subjected to increasing number (indicated next to each trace) of 66-Hz conditioning pulses (each to 0 mV for 5 ms from −100 mV). C, a family of tail currents, elicited by 10 nM permethrin, recorded (at −110 mV) from an oocyte expressing the wild-type Na⁺ channel. The oocyte was subjected to 100 conditioning pulses (each 5 ms in duration) at 66 Hz of increasing amplitude (millivolts; indicated next to each trace) from a holding potential of −100 mV (the dotted lines in A and B represent the holding current).

Fig. 6. M918T channels are weakly sensitive to permethrin. A, tail currents recorded (at −110 mV) from an oocyte expressing the M918T mutant in response to 1 μM (trace 1) and 10 μM permethrin (trace 2). The oocyte was subjected to the ATX-II protocol. In each trace, only the first 800 ms from the onset of depolarization is illustrated. The dotted line represents the holding current required to clamp the oocyte at −100 mV. B, concentration-response relationships for permethrin action on the single mutant M918T (■) and on the double mutant (M918T+L1014F) (▲) determined using the ATX-II protocol. Each datum point represents the mean of three experiments and the error bars are S.E.M. Each set of data were fitted with a Hill equation (solid line) (Table 2).
even into (Durell et al., 1998), the membrane bilayer, thus favoring the interaction of residues Met918 and Thr929 with pyrethroid partitioned into the membrane. It follows that interactions between these residues and pyrethroid would be enhanced when the channel is open, as shown by our studies, and that bound pyrethroid would stabilize the channel open state by holding the IIS4-S5 linker in its open channel conformation, thereby inhibiting deactivation. Durell et al. (1998) predicted that when the channel is open, S4 spans only the outer half of the transmembrane region, whereas S4-S5 spans the inner half. Using high-resolution nuclear magnetic resonance spectroscopy coupled with distance-geometry/simulated-annealing calculations to determine the three-dimensional structure of the S4-S5 segment of the D. melanogaster Shaker potassium channel, Ohlenschläger et al. (2002) confirmed the accuracy of this prediction. Durell et al. (1998) also predicted that for the channel to close, S4 and consequently S4-S5 must move inwards. According to Ohlenschläger et al. (2002), the structure of S4-S5 when the channel is open is a full α-helix, lying parallel to the pore axis. Therefore, he proposed that during channel closure the S4-S5 helix first breaks near its mid-region (which has a lower tendency for an α-helix conformation) and then the C-terminal part of S4-S5 becomes the N-terminal portion of the S5 helix. We propose that binding of pyrethroid to the open channel conformation of the IIS4-S5 linker slows the inwards movement of IIS4, a proposal that is consistent with a previous report that pyrethroids immobilize the gating charge of a Na⁺ channel (Salgado and Narahashi, 1993). The location of pyrethroid binding sites on the S4-S5 linker is supported by our studies of pyrethroid action on the rat brain IIA Na⁺ channel (Na⁺, 1.2) (Vais et al., 2000b): the sensitivity of this channel to pyrethroids is greatly increased by a single mutation (I874M) at a position equivalent to that of the house fly M918F mutation. Similar results have been obtained with other mammalian Na⁺ channel isoforms (i.e., Na⁺, 1.4) (Wang et al., 2001) and Na⁺, 1.8 (Soderlund and Lee, 2001). It seems clear that the cooperative binding of deltamethrin involves residues Met918 and Thr929 on the S4-S5 linker, because cooperativity is lost after resistance mutations of either of these residues. Leu1014 is not involved in cooperative binding of deltamethrin, because its mutation to L1014F reduces 

\[ K_a \]
but does not influence cooperativity.

The tail currents induced by both permethrin and deltamethrin are often preceded by a hook current. Hook currents are seen in the absence or presence of ATX-II, so they are unlikely to result from re-openings of inactivated channels per se or from the action of ATX-II. One possible explanation is that a hook current results from the reopening of fast and slow inactivated channels that contain trapped pyrethroid (Smith et al., 1997). This would explain why we found (data not shown) that the amplitude of a hook current is reduced after ATX-II application (i.e., when fast inactivation is inhibited). Studies of slow inactivation of insect Na⁺ channels have not yet been undertaken to test its possible involvement in generating hook currents.

The dependence of tail current time constant on deltamethrin concentration that was observed with the wild-type channel suggests complex channel deactivation kinetics, possibly related to the cooperative binding of this pyrethroid. A better understanding of this phenomenon is expected to arise from single channel investigations.

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References


