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Short communication

Effect of a fluvalinate-resistance-associated sodium channel mutation from varroa mites on cockroach sodium channel sensitivity to fluvalinate, a pyrethroid insecticide

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Abstract

Fluvalinate is a pyrethroid insecticide that is widely used in the control of the varroa mite (*Varroa destructor*), an ecto-parasite of the honeybee. Previously we identified four fluvalinate-resistance-associated mutations in the sodium channel gene of the varroa mite. One of the mutations caused a leucine (L) to proline (P) change at 1770 in the linker connecting domains III and IV of the sodium channel. Interestingly, at the position corresponding to the L to P mutation, all known insect (including honeybee) sodium channel proteins already naturally contain a P residue (e.g., P1577 in the cockroach sodium channel $BgNa_v$). To determine whether insect sodium channels are less sensitive to fluvalinate than arachnid sodium channels, we replaced P1577 with an L in a $BgNa_v$ variant ($BgNa_v1$-1) and examined the sensitivity of the recombinant channel to fluvalinate. The P1577L substitution did not alter the gating properties of the $BgNa_v1$-1 channel expressed in *Xenopus* oocytes. However, the $BgNa_v1\text{-}1^{P1577L}$ channel was five-fold more sensitive to fluvalinate compared with the $BgNa_v1$-1 channel. These results not only implicate the L to P mutation in fluvalinate resistance in varroa mites, but also suggest a possible contribution of L1770 to the higher sensitivity of varroa mites to fluvalinate than their insect hosts.

Keywords: *Para*; Voltage-gated sodium channel; Pyrethroid resistance; Knockdown resistance; Varroa mite

1. Introduction

Pyrethroid insecticides are a large class of synthetic analogs of the naturally occurring pyrethrum from the flower extracts of *Chrysanthemum* species. They are widely used to control many agriculturally and medically important arthropod pests. However, in the past two decades pest resistance to these compounds has become a global phenomenon, threatening the effective chemical control of human disease vectors, and agricultural and household arthropod pests. Pyrethroids exert their toxic effects primarily by altering gating properties of the sodium channel, which is essential for the generation and propagation of action potential in excitable cells (Narahashi, 1996). Pyrethroid resistance due to reduced neuronal sensitivity to these compounds, known as knockdown resistance (*kdr*), is one of the most important mechanisms by which insects develop pyrethroid resistance (Soderlund and Bloomquist, 1990). Research in the past 10 years provided clear evidence that mutations in the target site (sodium channel gene) are responsible for *kdr* and *kdr*-like resistance in many insect species (Dong, 2003; Soderlund and Knipple, 2003; Soderlund, 2005) (Table 1).

Insect sodium channels are large transmembrane proteins containing four-homologous domains (I–IV), each of which has six transmembrane segments (S1–6) (Loughney et al., 1989). It appears that both common and unique sodium channel mutations are associated with pyrethroid

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Table 1
Gating properties of wild-type and mutant $BgNa_v$ channels

Sodium channel	Activation		Inactivation		N
	$V_{1/2}\pm$SE	$k\pm$SE	$V_{1/2}\pm$SE	$K\pm$SE	
Wild-type	-23.5 ± 0.5	5.8 ± 0.1	-44.7 ± 0.3	5.3 ± 0.1	10
P1577L	-25.4 ± 0.6	4.9 ± 0.1	-44.8 ± 0.4	5.1 ± 0.1	11

resistance in different insect species. The *kdr* mutations that have been shown to reduce the insect sodium channel sensitivity to pyrethroids reside in different domains of the sodium channel, mostly within the sixth transmembrane segments or in the intracellular loops near the fifth and sixth segments (references in Soderlund, 2005).

The *kdr*-type resistance is not confined to insect pests. Extensive use of pyrethroids in controlling arachnid pests, such as the southern cattle ticks (*Boophilus microplus*) and varroa mites (*Varroa destructor*), an ectoparasite of the honeybee, has also led to the development of pyrethroid resistance in many arachnid populations. Surprisingly, none of the insect *kdr* mutations was detected in pyrethroid-resistant southern cattle ticks or varroa mites (He et al., 1999; Wang et al., 2002). A unique phenylalanine (F) to isoleucine (I) mutation in IIIS6 was identified in the pyrethroid resistant cattle ticks (He et al., 1999). This mutation when introduced into a cockroach sodium channel completely abolished the channel sensitivity to pyrethroids (Tan et al., 2005). The same mutation also reduced the sensitivity of a recombinant mammalian sodium channel to deltamethrin (Wang et al., 2001).

Fluvalinate is a pyrethroid insecticide widely used in the control of varroa mites. By comparing fluvalinate-resistant and fluvalinate-susceptible varroa mite populations, we previously found four sodium channel mutations in two fluvalinate-resistant varroa mite populations (Wang et al., 2002). These mutations include a phenylalanine (F) to leucine (L) mutation in the sixth segment of domain III (IIIS6), a leucine (L) to proline (P) mutation in the linker connecting domains III and IV, an isoleucine (I) to valine (V) mutation in IVS5, and a methionine (M) to isoleucine (I) mutation in IVS6. Interestingly, sequence comparison revealed that at the position equivalent to the varroa mite L1770P mutation, proline is found in all known insect sodium channels (Fig. 1), including *Drosophila melanogaster*, *Musca domestica*, *Blattella germanica*, *Heliothis virescens*, *Anopheles gambiae*, and *Apis mellifera*, although the residue corresponding to L1770 is quite variable in vertebrate sodium channels (e.g., F in rat $Na_v1.2$). This observation raises the possibility that insect sodium channels may be generally less sensitive to pyrethroids than arachnid sodium channels and that pyrethroid-resistant varroa mites have selected the L to P mutation to confer pyrethroid resistance. To test this hypothesis, we introduced this varroa mite sodium channel mutation into the wild-type cockroach $BgNa_v1$-1 sodium channel, expressed the recombinant channel in *Xenopus* oocytes, and evaluated the sensitivity of the recombinant channel to fluvalinate.

2. Materials and methods

As mentioned above, at the position corresponding to the mite L1770P mutation, the cockroach sodium channel already has a proline residue (P1577)1. Thus, a P1577L substitution was introduced into the wild-type sodium channel $BgNa_v1$-1, previously called KD1 (Tan et al., 2002a, b), by site-directed mutagenesis to generate $BgNa_v^{P1577L}$. For site-directed mutagenesis, a 1.4-kb *Eco47*III fragment containing P1577 was excised from $BgNa_v1$-1 and cloned into pAlter 1 (Promega Corp., Madison, WI). The point mutation was made using the Altered Sites II in vitro Mutagensis System (Promega Corp.) and confirmed by DNA sequencing. The mutated *Eco47*III fragment was excised from pAlter1 and cloned back into $BgNa_v1$-1 to produce the recombinant construct.

The procedures for oocyte preparation and cRNA injection were identical to those described by Tan et al. (2002b). For robust expression of the cockroach $BgNa_v1$-1 sodium channel, $BgNa_v1$-1 cRNA (1 ng) was co-injected into *Xenopus* oocytes with *D. melanogaster* tipE cRNA (1 ng), which is known to enhance the expression of insect sodium channels in oocytes (Feng et al., 1995).

The methods for electrophysiological recording, data analysis and measurement of channel sensitivity to pyrethroids were identical to those described previously (Tan et al., 2002a, b). The voltage-dependence of sodium channel conductance (G) was calculated by measuring the peak current at test potentials (V_t) ranging from -120 to $+60$ mV in 5-mV increments and dividing by (V_t-V_{rev}), where V_t is the test potential and V_{rev} is the reversal potential for sodium. Reversal potentials were determined from I–V curves. Peak conductance values were fitted with a Boltzmann equation of the form $G = 1-[1+\exp(V_t-V_{1/2})/k]^{-1}$, in which V_t is the potential of the voltage pulse, $V_{1/2}$ is the half-maximal voltage for activation, and k is the slope factor. The voltage-dependence of sodium channel inactivation was determined using 200-ms inactivating pre-pulses from a holding potential of -120 to $+40$ mV in 5-mV increments, followed by test pulses to -5 mV for 12 ms. The peak-current amplitude during the test depolarization was normalized to the maximum current amplitude, and plotted as a function of the pre-pulse potential. The data were fitted with a Boltzmann equation of the form $I = I_{max}[1+(\exp(V_p-V_{1/2})/k)]^{-1}$, in which I_{max} is the maximal current evoked, V_p is the potential of the voltage pulse, $V_{1/2}$ is the voltage at which 50% of the current is inactivated (the midpoint of the inactivation curve), and k is the slope factor.

To record fluvalinate-induced tail currents, we applied a 100-pulse train of 5-ms depolarization from -120 to -10 mV to increase the availability of open channels (Vais et al., 2000). The method for fluvalinate application was

identical to that described in Tan et al. (2002a, b). The working concentration was prepared in ND96 recording solution immediately prior to experiments. The concentration of DMSO in the final solution was <0.5%, which had no effect on sodium channels. The fluvalinate-induced tail currents were measured 10 min after toxin application using the protocol identical to that in Tan et al. (2002a, b). Percentages of channels modified by fluvalinate were calculated using the equation $M = \{[I_{tail}/(E_h - E_{Na})]/[I_{Na}/(E_t - E_{Na})]\} \times 100$, where I_{tail} is the maximal tail current amplitude, E_h, E_{Na} and E_t are the holding potential, reversal potential, and test potential, respectively. I_{Na} is the amplitude of the peak current during depolarization before fluvalinate exposure. Dose-response curves were fitted to the Hill equation: $M = M_{max}/\{1 + (EC_{50}/[\text{pyrethroid}])^n\}$, in which [pyrethroid] represents the concentration of fluvalinate and EC_{50} represents the concentration of fluvalinate that produced the half-maximal effect, n represents the Hill coefficient, and M_{max} is the maximal percentage of sodium channel modified. Because voltage-clamp fails at higher fluvalinate concentrations due to large leakage currents, we cannot obtain the upper portion of the dose-response curve. EC_{20} values were used to compare channel sensitivities among wild-type and mutant channels.

3. Results and discussion

P1577 in $BgNa_v1$-1 is located in the short conserved intracellular linker connecting domains III and IV, which is critical for fast inactivation (Fig. 1). To determine whether the P1577L mutation alter the channel gating, we expressed the $BgNa_v^{P1577L}$ channel in *Xenopus* oocytes and examined the channel gating properties. The $BgNa_v^{P1577L}$ channel produced sufficient currents in *Xenopus* oocytes for

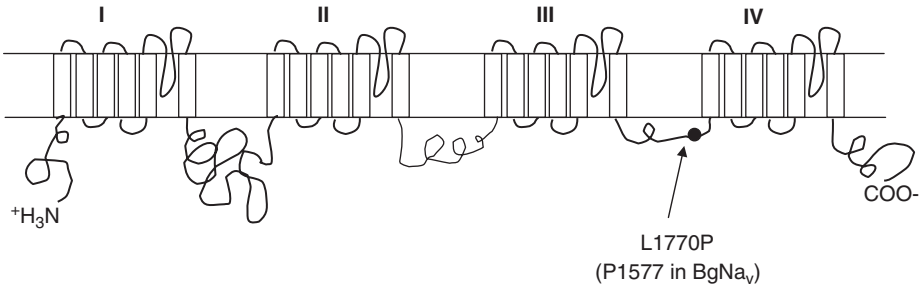

Fig. 1. Schematic drawing of the sodium channel topology indicating the position of the pyrethroid resistance-associated L1770P mutation in varroa mites (numbered according to the published amino acid sequence of VmNa; GenBank accession number: AY259834, Wang et al., 2003). P1577 is the corresponding amino acid residue in the cockroach $BgNa_v1.1$ channel (GenBank accession number: U73583).

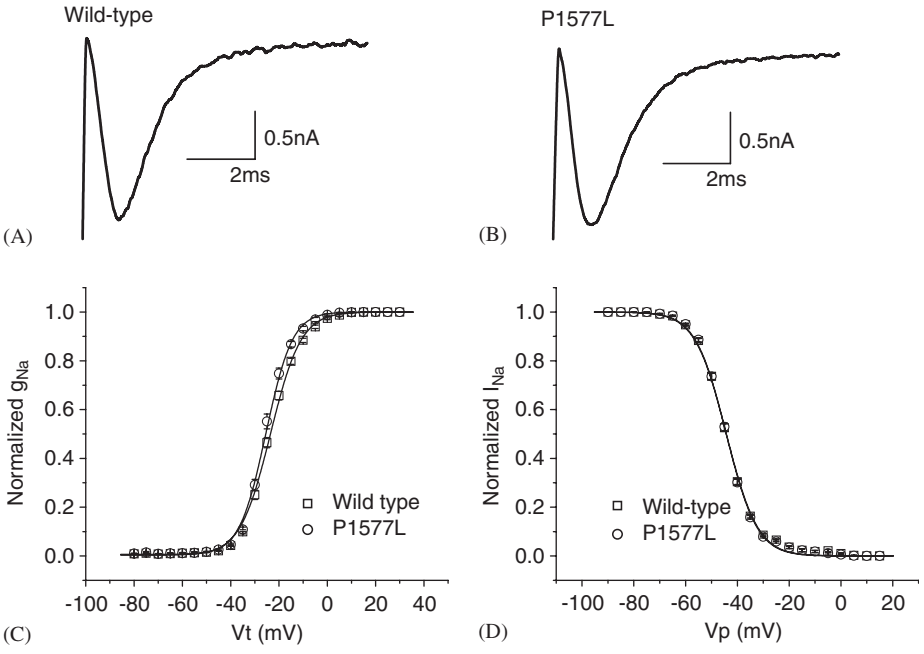

Fig. 2. Gating properties of wild-type and mutant $BgNa_v1$-1^{P1577L} sodium channels. Sodium current traces from the wild-type (A) or mutant $BgNa_v1$-1^{P1577L} (B) channel. The sodium currents were elicited by a 20 ms depolarization to 0 mV. (C) Voltage-dependence of activation. (D) Voltage-dependence of steady-state inactivation. The protocols for the voltage-dependence of activation and inactivation are described in Section 2.

electrophysiological and pharmacological analyses. The $BgNa_v^{P1577L}$ channel activated (i.e., open) and inactivated (i.e., closed) rapidly like the wild-type $BgNa_v1$-1 channel, indicating that the P1577L mutation did not alter the fast inactivation kinetics (Fig. 2A and B). The $BgNa_v^{P1577L}$ channel also exhibited the voltage-dependence of activation and steady-state inactivation similar to those of the wild-type channel (Fig. 2C and D). Therefore, the P1577L mutation did not alter these gating properties.

Pyrethroids inhibit deactivation and inactivation of sodium channels, resulting in prolonged opening of sodium channels. Prolonged channel opening leads to the appearance of a large tail current associated with depolarization. The amplitude and decay of the tail current are used to quantify the degree of modification of sodium channels by pyrethroids. In oocytes expressing the wild-type $BgNa_v1$-1 channel, fluvalinate induced a detectable tail current at 0.1 μM (Fig. 3A). However, a significantly larger tail current was found in oocytes expressing $BgNa_v^{P1577L}$ channels (Fig. 3B). To quantify the modification of sodium channels by fluvalinate, the tail current amplitude was normalized by the peak current and converted to the percentage of channel modification by fluvalinate using the equation described in Section 2. Significantly greater modification by fluvalinate was observed for the $BgNa_v^{P1577L}$ channel, compared with the wild-type $BgNa_v$ 1-1 (Fig. 3C). The $BgNa_v^{P1577L}$ channel was five-fold more sensitive to fluvalinate than the wild-type $BgNa_v$ 1-1 channel. Thus, the P1577L substitution made the recombinant channel more sensitive to fluvalinate. A five-fold difference in channel sensitivity to fluvalinate is comparable to that mediated by the *kdr* mutation L993F (Tan et al., 2002a).

It is interesting that at the position corresponding to L1770 in varroa mites, an L is also found in the sodium channels from the southern cattle tick (He et al., 1999) and the human scabies mite (*Sarcoptes scabiei* var *hominis*) (Pasay et al., 2006), the only two other arachnids whose sodium channel sequences are available. In contrast, as mentioned above, proline is present in all known sodium channel proteins in insects. Our results therefore provide the first molecular evidence that insect sodium channels may be generally less sensitive to pyrethroids than arachnid sodium channels. Indeed, a recent toxicological study has shown that fluvalinate was 500-fold more toxic to varroa mites than to honeybees (Santiago et al., 2000). Our finding is reminiscent of the work by Vais et al. (2000) which showed that a single residue (M918 in the house fly Vssc1 sodium channel) is partially responsible for the higher pyrethroid sensitivity of an insect sodium channel than that of a mammalian sodium channel. Specifically, substitution of I with M at the corresponding position in the rat sodium channel $Na_v1.2$ enhanced $Na_v1.2$ sensitivity to pyrethroids (Vais et al., 2000). Future functional characterization of arachnid sodium channels will be necessary to further confirm the differential sensitivity of insect and arachnid sodium channels to pyrethroids.

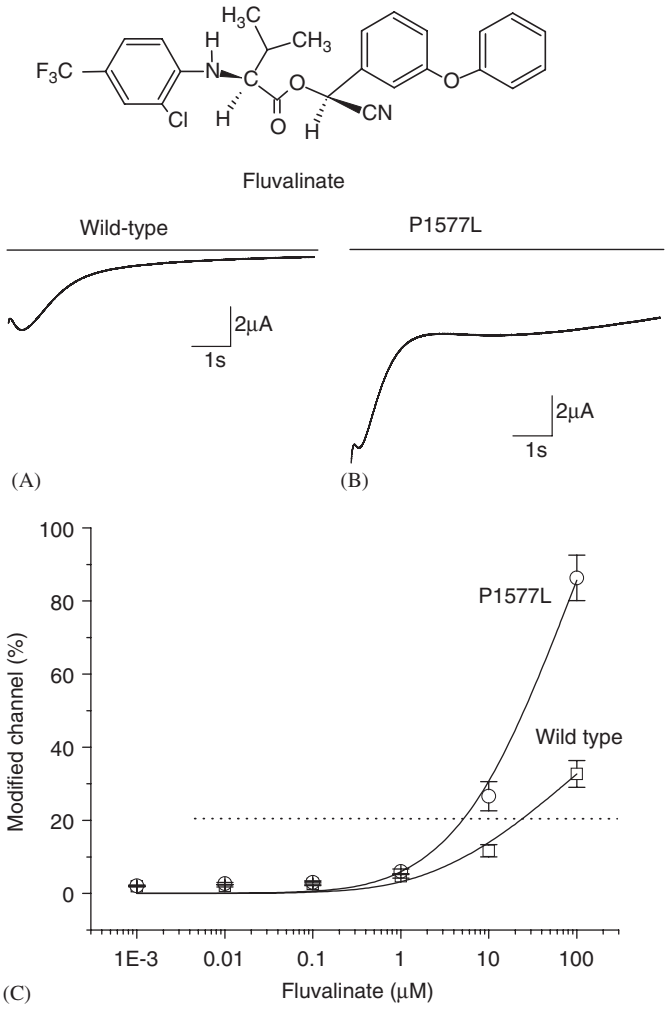

Fig. 3. Sensitivity of wild-type and mutant $BgNa_v1$-1^{P1577L} sodium channels to fluvalinate. A and B. Tail currents induced by fluvalinate from oocytes expressing the wild-type (A) and the mutant (B) channels. (C) Percentages of channel modification by fluvalinate. The protocol for the tail current recording and the method for quantitative analysis of pyrethroid modification of sodium channels are described in Section 2. EC_{20}s of fluvalinate are 22.6 ± 1.2 μM ($n = 5$), and 4.8 ± 1.2* μM ($n = 4$), respectively, for the wild-type and mutant channels. *Significant difference compared with the wild-type channel by student's *t*-test ($p < 0.05$).

Prior to this study, most *kdr* and super-*kdr* mutations are found located in the transmembrane segments S6 in sodium channel homologous domains I, II or III; in S5 of domain II, in the intracellular loop connecting domains I and II; and in a small intracellular loop connecting S4 and S5 in domain II (Soderlund, 2005). Recent studies suggest that the pyrethroid-binding site is composed of residues within the sixth segments of domain II and III and the intracellular loops near the transmembrane segments. For example, mutations in IIS6 and IIIS6 have been shown to reduce pyrethroid binding to the sodium channel (Tan et al., 2005). Electrophysiological and modeling studies also implicate a super-*kdr* mutation (M918T in the small intracellular loop connecting IIS4 and IIS5) in affecting pyrethroid binding (Vais et al., 2000, 2003, O'Reilly et al.,

2006). Although our results suggest that the L1770P mutation is involved in the pyrethroid resistance in varroa mites, how L1700 contributes to the action of pyrethroids on the sodium channel remains to be determined. P1577L is close to the C-terminal end of the intracellular loop connecting domains III and IV (six amino acid residues upstream of IVS1). Judging by its unique position, it seems unlikely that this position could be part of the pyrethroid-binding site. Because the P1577L mutation did not alter the channel gating properties it is also unlikely that the effect of the P1577L is exerted through modifying sodium channel gating. However, the P1577L mutation could affect pyrethroid interaction with the sodium channel by a novel mechanism (e.g., by altering the pyrethroid-induced conformational change that is required for pyrethroid action). It is clear from this and previous studies that differential sensitivities of various sodium channels to pyrethroids are rich resources for elucidating the molecular basis of pyrethroid action on the sodium channel.

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