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Alanine to valine substitutions in the pore helix IIIP1 and linker-helix IIIL45 confer cockroach sodium channel resistance to DDT and pyrethroids

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Abstract

Pyrethroid insecticides exert toxic effects by prolonging the opening of voltage-gated sodium channels. More than 20 sodium channel mutations from arthropod pests and disease vectors have been confirmed to confer pyrethroid resistance. These mutations have been valuable in elucidating the molecular interaction between pyrethroids and sodium channels, including identification of two pyrethroid receptor sites. Previously, two alanine to valine substitutions, one in the pore helix IIIP1 and the other in the linker-helix connecting S4 and S5 in domain III (IIIL45), were found in *Drosophila melanogaster* mutants that are resistant to DDT and deltamethrin (a type II pyrethroid with an α -cyano group at the phenylbenzyl alcohol position, which is lacking in type I pyrethroids), but their role in target-site-mediated insecticide resistance has not been functionally confirmed. In this study, we functionally examined the two mutations in cockroach sodium channels expressed in *Xenopus laevis* oocytes. Both mutations caused depolarizing shifts in the voltage dependence of activation, conferred DDT resistance and also resistance to two Type I pyrethroids by almost abolishing the tail currents induced by Type I pyrethroids. In contrast, neither mutation reduced the amplitude of tail currents induced by the Type II pyrethroids, deltamethrin or cypermethrin. However, both mutations accelerated the decay of Type II pyrethroid-induced tail currents, which normally decay extremely slowly. These results provided new insight into the molecular basis of different actions of Type I and Type II pyrethroids on sodium channels. Computer modeling predicts that both mutations may allosterically affect pyrethroid binding.

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Graphical abstract

1. Introduction

Voltage-gated sodium channels are essential for the initiation and propagation of action potentials in neurons and other excitable cells. Sodium channel α -subunits have four homologous repeat domains (I-IV), each possessing six transmembrane segments (S1-S6; Fig. 1A). In each domain, the S1-S4 segments constitute the voltage-sensing module. The segments S5 and S6 from the four domains, in addition to the four membrane-reentrant P-loops (P1 and P2) that connect the S5 and S6 segments, form the pore module. Because of their critical roles in electrical signaling, sodium channels are effective targets of a variety of naturally occurring and synthetic neurotoxins including pyrethroid insecticides. Both pyrethroids and DDT inhibit channel deactivation and inactivation and stabilize the open state of sodium channels causing prolonged channel opening (Bloomquist, 1996; Bloomquist and Soderlund, 1988; Narahashi, 2000; Narahashi et al., 1992; Silver et al., 2014; Vijverberg and van den Bercken, 1982; Vijverberg et al., 1982).

Pyrethroids are grouped into two categories (Type I and Type II) based on their distinct poisoning symptoms in rats, effects on nerve preparations, and chemical structures (Gammon et al., 1981; Lawrence and Casida, 1982; Narahashi, 1986). Type I pyrethroids lack an α -cyano group, which is present at the phenylbenzyl alcohol moiety of Type II pyrethroids. Type I pyrethroids cause repetitive discharges in response to a single stimulus, whereas Type II pyrethroids cause membrane depolarization accompanied by suppression of the action potential (Gammon et al., 1981; Lund and Narahashi, 1981; Narahashi, 1986). Earlier electrophysiological studies using nerve preparations (Lund and Narahashi, 1982; Narahashi et al., 1992; Vijverberg and van den Bercken, 1982) clearly showed that the decay of tail currents induced by Type II pyrethroids are at least an order of magnitude slower than those induced by Type I pyrethroids, indicating that Type II pyrethroids inhibit the deactivation of sodium channels to a much greater extent than Type I pyrethroids. More recent studies on the effects of pyrethroids on insect and mammalian sodium channels expressed in *Xenopus laevis* oocytes have confirmed and extended these earlier findings (Soderlund, 2010, 2012). The quantitative differences in the kinetics of tail current decay

between the two types of pyrethroids likely contribute, in part, to the differences in actions of Type I and II pyrethroids observed *in vivo* (Narahashi, 1986).

DDT and pyrethroids have been used extensively in controlling arthropod pests and disease vectors. Although the use of DDT is largely banned, DDT is still one of the recommended insecticides for malaria control in Africa. A major obstacle to the effective use of these compounds is the emergence of resistance. One major mechanism of DDT and pyrethroid resistance is known as knockdown resistance (kdr) (Soderlund and Bloomquist, 1990). kdr and kdr-like resistance have been documented globally; and mutations in various regions of insect voltage-sensitive sodium channels are reported to be associated or responsible for kdr in diverse arthropod pests and disease vectors (Rinkevich et al., 2013; Soderlund, 2005; Dong et al., 2014).

Identification of kdr mutations not only provides precise molecular markers for rapidly assessing the frequency of resistance alleles in field populations, but has also proven to be extremely valuable for elucidating the molecular identity of pyrethroid receptor sites, PyR1 and PyR2 (Dong et al., 2014; Du et al., 2009; Du et al., 2013b; O'Reilly et al., 2006; Usherwood et al., 2007). The PyR2 and PyR1 sites are located, respectively, in domain interfaces I/II and II/III and are arranged quasi-symmetrically. At each site, pyrethroids bind between four helices: L45, S5 and S6 from a given domain and helix S6 from another domain which at the extracellular view of the channel is a clockwise neighbor of the given domain (Du et al., 2015).

In the model insect, *Drosophila melanogaster*, earlier molecular and genetic analyses of a large collection of mutants exhibiting temperature-sensitive paralytic phenotypes led to the identification of *para* (later named as DmNa_v), the sodium channel gene in *D. melanogaster* (Loughney et al., 1989). Remarkably, several of these temperature-sensitive mutants were resistant to DDT and deltamethrin, a type II pyrethroid (Pittendrigh et al., 1997). Subsequent molecular analysis uncovered several mutations in DmNa_v that were associated with DDT and deltamethrin resistance (Fig. 1; Pittendrigh et al., 1997). Two of these mutations, A1548V and A1648V (Pittendrigh et al., 1997), were located in the pore helix of domain III (IIIP1) and the linker helix connecting S4 and S5 in domain III (IIIL45); and to facilitate sequence comparison among sodium channels from various species, the mutations are also named as A^{3k10}V and A^{3p47}V (see Figure 1 legend for the nomenclature). Whether or not these mutations contribute to resistance to DDT and pyrethroids, however, was not determined. In this study, we introduced mutations A^{3k10}V and A^{3p47}V into a German cockroach (*Blattella germanica*) sodium channel (BgNa_v1-1a) and examined the insecticide sensitivity of the resultant mutant channels in *Xenopus* oocytes. Remarkably, both mutations confer differential effects on the action of Type I and Type II pyrethroids, providing new insight into the molecular basis of actions of pyrethroids on sodium channels.

2. Materials and Methods

2.1. Site-directed mutagenesis

BgNa_v1-1a (pyrethroid-sensitive), a German cockroach voltage-gated sodium channel variant, was mutagenized to generate alanine to valine mutants A^{3k10}V and A^{3p47}V (Fig. 1).

Site-directed mutagenesis was performed by PCR using mutant primers and Phusion High-Fidelity DNA Polymerase (NEB, Ipswich, MA). All mutagenesis results were verified by DNA sequencing.

2.2. Expression of BgNa_v1-1a sodium channels in *Xenopus* oocytes

The procedures for oocyte preparation, cRNA synthesis and injection were identical to those described previously (Tan et al., 2002). cRNA was prepared by *in vitro* transcription with T7 polymerase using the mMESSAGE mMACHINE® high yield capped RNA kit (Ambion, Austin, TX). For robust expression of the BgNa_v1-1a sodium channels, BgNa_v1-1a cRNA was coinjected into oocytes with *Drosophila melanogaster* tipE cRNA (1:1 molar ratio) which enhances the expression of insect sodium channels in oocytes (Feng et al., 1995; Warmke et al., 1997).

2.3. Electrophysiological recording and analysis

Sodium currents were recorded by using standard two-electrode voltage clamping. Methods for electrophysiological recording and data analysis were similar to those described previously (Tan et al., 2005).

The voltage dependence of sodium channel conductance (*G*) was calculated by measuring the peak current at test potentials ranging from -80 to +65 mV in 5 mV increments and divided by (*V* - *V*_{rev}), where *V* is the test potential and *V*_{rev} is the reversal potential for sodium ions. Peak conductance values were normalized to the maximal peak conductance (*G*_{max}) and fitted with a two-state Boltzmann equation of the form:

$$G/G_{max} = [1 + \exp(V - V_{1/2})/k]^{-1}$$

in which *V* is the potential of the voltage pulse, *V*_{1/2} is the voltage for half-maximal activation, and *k* is the slope factor.

The voltage dependence of sodium channel inactivation was determined by using 100 milliseconds inactivating prepulses ranging from -120 to -10 mV in 5 mV increments from a holding potential of -120 mV, followed by test pulses to -10 mV for 20 milliseconds. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude and plotted as a function of the prepulse potential. Data were fitted with a two-state Boltzmann equation of the form:

$$I/I_{max} = [1 + (\exp(V - V_{1/2})/K)]^{-1}$$

in which *I* is the peak sodium current, *I*_{max} is the maximal current evoked, *V* is the potential of the voltage prepulse, *V*_{1/2} is the half maximal voltage for inactivation, and *k* is the slope factor.

2.4. Insecticides

(1R,3R, α -S)-deltamethrin and cypermethrin (mixture of isomers) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA); (1R)-cis-permethrin and DDT were purchased from Chem Service (Chem Service, West Chester, PA, USA); (1R)-cis-NRDC 157 was a gift from Bhupinder Khambay (Rothamsted Research, Harpenden, United Kingdom). The purities of different pyrethroids range from 94.3% to 99.6%.

2.5. Measurement of tail currents induced by pyrethroids

The method for application of pyrethroids in the recording system was identical with that described previously (Tan et al., 2002). The effects of pyrethroids were measured 10 minutes after their application. Pyrethroid-induced tail currents were recorded during a 100-pulse train of 5 milliseconds step depolarizations from -120 to 0 mV with 5 milliseconds interpulse intervals (Vais et al., 2000). The percentage of channels modified by pyrethroids was calculated using the following equation (Tatebayashi and Narahashi, 1994):

$$M = \{ [I_{tail} / (E_h - E_{Na})] / [I_{Na} / (E_t - E_{Na})] \} \times 100$$

where I_{tail} is the maximal tail current amplitude, E_h is the potential to which the membrane is repolarized, E_{Na} is the reversal potential for sodium current determined from the current-voltage curve, I_{Na} is the amplitude of the peak current during depolarization before pyrethroid exposure, and E_t is the potential of the step depolarization.

In order to evaluate the effect of amino acid substitutions on tail current decays, we determined integral modification (M_I) as developed by Usherwood et al. (2007).

$$M_I = \{ [I_{tail,integ} / (E_h - E_{Na})] / [I_{Na} / (E_t - E_{Na})] \} \times 100$$

where $I_{tail, integ}$ is the total charge flow during a tail current which is fitted by one or two exponential components. E_h is the potential to which the membrane is repolarized, E_{Na} is the reversal potential for sodium current determined from the current-voltage curve, I_{Na} is the amplitude of the peak current during depolarization before pyrethroid exposure, and E_t is the potential of the step depolarization.

2.5. Modeling

Sequence alignment of $K_v1.2$ (a mammalian potassium channel) (Long et al., 2005), Na_vAb (a voltage-gated sodium channel from *Arcobacter butzleri*) (Payandeh et al., 2011) and $BgNa_v1-1a$ channels is shown in Figure 1. Residues that contribute to pyrethroid receptors PyR1 and PyR2, as well as A^{3k10} and A^{3p47} are highlighted. Within the pore module, the sequences of the cockroach sodium channel $BgNa_v1-1a$ and mosquito sodium channel $AaNa_v1-1$ (a voltage-gated sodium channel from *Aedes. aegypti*) are very similar and are identical at the known pyrethroid binding sites, PyR1 and PyR2. Therefore, we have used our model of the $AaNa_v1-1$ channel (Du et al., 2015), which is based on the X-ray structure of the open potassium channel, $K_v1.2$ (Long et al., 2005), to visualize residues A^{3k10} and A^{3p47} that have been mutated in this study, as well as their 3D neighbors. Molecular images

were created using the PyMol Molecular Graphics System, Version 0.99rc6 (Schrödinger, LLC, New York, NY).

3. Results and Discussion

3.1. Mutations A^{3k10V} and A^{3p47V} alter the gating of BgNa_v1-1a channels

We introduced A^{3k10V} or A^{3p47V} mutations individually into BgNa_v1-1a, an insecticide sensitive cockroach sodium channel, to generate two mutants, A^{3k10V} or A^{3p47V}.

BgNa_v1-1a and the two mutant channels were expressed in *Xenopus* oocytes and examined for gating and sensitivity to DDT, permethrin (PMT), or deltamethrin (DMT). Notably, both mutations shifted the voltage-dependence of channel activation in the depolarizing direction by 13-15 mV (Table 1 and Fig. 2). Furthermore, the A^{3k10V} mutation also shifted the voltage dependence of inactivation in the hyperpolarizing direction by 6 mV (Table 1 and Fig. 2).

3.2. Mutations A^{3k10V} and A^{3p47V} abolished tail currents induced by two Type I pyrethroids, but only accelerated the decay of tail currents induced by two Type II pyrethroids

To evaluate the effects of pyrethroids on the mutant sodium channels, we used a multiple short depolarizations to elicit pyrethroid-induced tail currents. These pyrethroid-induced tail currents associated with repolarization under voltage-clamp conditions reflect the prolonged opening of pyrethroid-modified sodium channels. The amplitude of pyrethroid-induced tail currents are commonly used to quantify the potency of pyrethroid modification of sodium channels using the method developed by Tatebayashi and Narahashi (1994).

As shown in Fig. 3A and B, no tail currents were detected in the absence of permethrin (PMT) (control) or deltamethrin (DMT) (control) in all BgNa_v1-1a and mutant channels. Both PMT and DMT induced tail currents in a dose-dependent manner from oocytes expressing BgNa_v1-1a channels (Fig. 3A and B). DMT inhibited the deactivation of sodium channels to a greater extent than PMT. As expected, the large tail currents induced by PMT and DMT at 1 μM exhibited very different kinetics. PMT-induced tail currents of BgNa_v1-1a channels decayed rapidly, which returned to the baseline within 2 seconds of the 8-second repolarization, whereas DMT-induced tail currents decayed much more slowly and a portion of the tail current remained at the end of the 8-second repolarization. In contrast, the amplitude of PMT-induced tail currents was almost abolished for both mutant channels, and the percentage of channel modification by PMT on the mutant channels was less than 8% compared to 94% on wild-type channels (Fig. 3A). These results indicated that both mutations reduced BgNa_v1-1a channel sensitivity to PMT. However, neither mutation reduced the amplitude of the tail currents induced by DMT and altered the sensitivity of BgNa_v1-1a channels to DMT (Fig. 3B).

Although the amplitude of DMT-induced tail currents was not reduced by the mutations, the DMT-induced tail currents in the mutants decayed faster than in the wildtype channels (Fig. 3B). We took both the amplitude and rate of decay into consideration in evaluating the potency of pyrethroid modification of sodium channels using a method developed by

Usherwood et al. (2007). As shown in Table 2, both mutations reduced the levels of the integral channel modification by DMT by 1.5-1.8-fold. We did not compare the decay of PMT-induced tail currents between BgNa_v1-1a and A^{3k10V} or A^{3p47V} mutant channels since the tail currents were almost abolished by the mutations.

We then examined the effects of two more pyrethroids on these mutant channels. Cypermethrin (CPMT) is a Type II pyrethroid, which differs structurally from PMT only by the presence of the α -cyano group. NRDC 157 is a DMT analogue lacking the α -cyano group. As shown in Fig. 4, the effects of the two mutations on the tail current amplitudes induced by NRDC 157 were similar to those by PMT; but there was no reduction in the amplitude of CPMT-induced tail currents. The decay of CPMT-induced tail currents, however, was accelerated by both mutations (Fig. 4 and Table 2). These mutational effects are consistent with those from PMT/DMT in Fig. 3, suggesting that the effects of the two mutations are pyrethroid type-specific.

This finding is the first reported case where sodium channel mutations alter the decay of pyrethroid-induced tail currents, but not the amplitude of the tail currents. However, it seems that the effects of these two mutations on the action of Type II pyrethroids were not as drastic as their effects on the action of Type I pyrethroids. The accelerated decay of DMT-modified sodium channels is likely responsible for the reported low level of deltamethrin resistance of temperature-sensitive paralytic mutant flies (Pittendrigh et al., 1997). Although the susceptibility of the paralytic mutants to permethrin or other Type I pyrethroids was not determined in that study, the two mutations are expected to also confer fly resistance to permethrin and other Type I pyrethroids.

3.3. Both mutations also confer BgNa_v1-1a channel resistance to DDT

DDT induces extremely small and fast decaying tail currents in the wild-type channels, even at concentrations of 100 μ M (Du et al., 2016). Because DDT also inhibits inactivation, we also assessed the channel sensitivity to DDT by determining the percentage of inhibition of inactivation. Figure 5 shows representative current traces from BgNa_v1-1a, A^{3k10V} and A^{3p47V}, and the percentages of inhibition of channel inactivation by DDT. Clearly, DDT caused less channel modification in the two mutants than in the wild-type channels, indicating that these two mutant channels are also resistant to DDT, which is consistent with the bioassay results from Pittendrigh et al. (1997).

3.4. Predictions from computational modeling

The different effects on the action of Type I and Type II pyrethroids by these two mutations are interesting, because although both Type I and Type II pyrethroids are sodium channel gating modifiers, they cause some distinct effects on sodium channel gating. However, the different effects caused by these two mutations on the action of type I and II pyrethroids are interesting because the molecular basis of differential gating modifications by pyrethroids remain elusive.

Here we suggest how these two mutations may alter the interactions of pyrethroids with cockroach sodium channels based on the homology modeling of cockroach sodium

channels, which was used to map pyrethroid receptor sites from our previous studies (Du et al., 2013b; Du et al., 2015).

3.4.1. Mutations A^{3k10}V and A^{3p47}V may shift helices L45-S5 in domain III away from the S6 bundle—

The PyR1 and PyR2 receptors demonstrate rotational pseudosymmetry around the pore axis (Du et al., 2015): rotation of the channel 90 degrees around the pore axis moves PyR1 into the place of PyR2. Whether or not additional pyrethroid binding site(s) are located in other domain interface(s) is currently unknown. Mutations A^{3k10}V and A^{3p47}V are found in domain III, far from helix IIIS6 that contributes to PyR1. In the K_v1.2-based model of the open BgNa_v1-1a channel (Fig. 6A and B) the methyl group of A^{3k10} closely approaches the cytoplasmic end of helix IIIS6 and makes a knob-into-the-hole contact with the C^βH₂ group of D³ⁱ²⁸ and C^αH group of N³ⁱ²⁹ (Fig. 6C). A ligand is unlikely to squeeze between these three groups implying that the A^{3k10}V mutation indirectly affects the action of pyrethroids. Since the interface between helices IIIL45 and IIIS6 is small, mutation A^{3k10}V could shift the helices apart from each other. Helix IIIS6, which interacts with four different helices (IIS5, IIS6, IVS6, and IIIL45) is less mobile than helix IIIL45, which interacts with only IIIS6 and IVS6. Therefore, mutation A^{3k10}V likely shifts helix IIIL45 away from the S6 bundle.

The A^{3p47} sidechain closely approaches helices IIS5 and IIIS6 at the extracellular half of the pore module (Figure. 6A and B) and fits between the sidechains of F^{3o16}, W^{3o17} and F³ⁱ¹⁰ (Fig. 6D). The interface between helices IIIP1, IIS5, and IIIS6 appears to be too tight to accommodate a ligand, implying that A^{3p47} is unlikely to directly interact with a pyrethroid molecule. Valine substitution A^{3p47}V would sterically clash with the backbone between F^{3o16} and W^{3o17} and shift IIS5 from IIIP1 and IIIS6. The opposite shift of IIIP1 towards the pore axis is unlikely because helices in the P-loop domain are tightly packed. Indeed, comparison of X-ray structures of different P-loop channels shows that mutual disposition of helices in the extracellular half of the pore module is much more conserved than at in the intracellular half.

Thus, both valine substitutions A^{3k10}V and A^{3p47}V would shift helices IIIL45 and IIS5 farther from the pore axis and the S6 bundle. Helices IIIL45 and IIS5 are continuations of each other and form a single kinked-helix segment III_L45-S5. Therefore, either of the two valine substitutions would shift the segment from the pore axis. The results from our gating analysis of the two mutants are consistent with this prediction as both A^{3k10} and A^{3p47} mutations alter the voltage dependence of channel activation.

3.4.2. How the III_L45-S5 shift may affect binding of pyrethroids?—

More than 20 mutations have been confirmed to reduce the pyrethroid sensitivity of insect sodium channels expressed in the *Xenopus laevis* oocyte expression system (Dong et al., 2014; Du et al., 2013b; Rinkevich et al., 2013). So far, most of the kdr mutations that have been mapped to PyR1 or PyR2 all reduced the amplitude of tail currents induced by both Type I and Type II pyrethroids to various degrees with the exception of one kdr mutation in *Aedes aegypti*, F1269C (F³ⁱ¹³C) in IIIS6 (Kawada et al., 2009), which did not reduce the amplitude of three type II pyrethroids examined including DMT (Hu et al., 2011). The two mutations examined in this study do not belong to either the PyR1 or PyR2 sites. Other kdr mutations

beyond the known pyrethroid binding sites have also been reported. For example, recently we proposed that I^{2o17}V, a *kdr* mutation found in esfenvalerate-resistant pollen beetle populations (Wrzesinska et al., 2014), is rather far from the channel-bound esfenvalerate, whereas I^{2o17} forms a close contact with L^{2p47} (Du et al., 2016). We further suggested that mutation I^{2o17}V would shift helix IIS5 towards helices IIP and IIS6, thus tightening the domain II/III interface and deforming the PyR1 receptor site where esfenvalerate binds (Du et al., 2016). Contacts A^{3p47} : W^{3o17} (Fig. 6 D) and L^{2p47} : I^{2o17} (Fig. 9A in (Du et al., 2016)) are at symmetric locations. The fact that two *kdr* mutations (A^{3p47}V and I^{2o17}V) are found at symmetric intra-domain contacts (2p47 : 2o17 and 3p47 : 3o17) deserves mentioning. It is also interesting that both *kdr* mutations appear to affect the binding of pyrethroids indirectly (allosterically).

Among the four sodium channel mutations identified in *Drosophila* mutants (Pittendrigh et al., 1997); the I265N mutation (also known as I^{1k12}N) in the linker helix L45 in domain II, was recently shown to cause resistance to DDT, PMT, and DMT in a variant of DmNa_v in *Xenopus* oocytes (Rinkevich et al., 2015). The I^{1k12}N mutation appeared to allosterically deform pyrethroid receptor PyR2 resulting in reduced pyrethroid binding (Rinkevich et al., 2015). Helices IIIL45 and IIS5 do not belong to PyR1 or PyR2. Although the mutations likely allosterically modify the binding of pyrethroids to the PyR1 site, we cannot exclude the possible existence of a third pyrethroid-binding site considering the positions of these two mutations are at the lipid-exposed III/IV domain interface, analogous to the PyR1 and PyR2 sites. The shift of segment III_L45-S5 from the pore axis and thus from IVS6 would deform the possible pyrethroid binding site in the III/IV interface.

3.4.3. Why mutations A^{3k10}V and A^{3p47}V affect the action of PMT and NRDC 157 stronger than the action of DMT and CPMT?—Earlier we docked DMT, 1R-cis-PMT and 1S-cis-PMT in PyR2, and found that the binding modes of the three ligands are similar, but not identical (Du et al., 2013b). In particular, as compared to PMT, DMT binds deeper between helices IL45, IS6 and IIS6 and occurs farther from IIS5. A possible cause is closely spaced polar residues at the cytoplasmic third of the IS6/IIS6 interface (Tikhonov et al., 2015), which may attract the α-cyano group of deltamethrin. Polar groups are also seen at the cytoplasmic third of the IIS6/IVS6 interface. If binding modes of pyrethroids in the III/IV interface somehow resemble those in the I/II interface, PMT and NRDC 157, which lack the α-cyano group, would bind more superficially, and interact with IIS5 more strongly as compared with DMT and CPMT. In this case, the shift of the III_L45-S5 segment should exert stronger effects on the action of PMT and NDRC 157 as seen from our experiments (Figs. 3 and 4).

In conclusion, functional characterization of two sodium channel mutations that were identified from *Drosophila* temperature-sensitive paralytic mutants revealed that both mutations alter gating of channels and their sensitivities to DDT and pyrethroids, but had distinct effects on the action of Type I and Type II pyrethroids. Furthermore, the analysis from our computational modeling predicts that the two mutations allosterically modify the binding and action of DDT and pyrethroids on BgNa_v1-1a channels, which are just first indications that pyrethroids may bind in the III/IV interface. Further molecular modeling, mutational, and electrophysiological studies are necessary to discover if a third pyrethroid

receptor is located in the III/IV interface of sodium channels and elaborate its atomic model, as well as to more completely understand the molecular basis of different effects of Type I and Type II pyrethroids on sodium channels.

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Highlights

- Two alanine to valine substitutions were associated with pyrethroid resistance
- One mutation is in the pore helix IIIP1 and the other is in the linker-helix connecting S4 and S5 in domain III.
- Both mutations reduced the amplitude of tail currents induced by Type I pyrethroids.
- Both mutations accelerated the decay of tail currents induced by Type II pyrethroids.

Figure 1.

A. Sodium channel topology indicating two mutations from the temperature-sensitive paralytic mutants of *D. melanogaster* that were resistant to DDT and deltamethrin (Pittendrigh et al., 1997). We use a residue-labeling scheme that is universal for P-loop channels (Du et al., 2013a; Zhorov and Tikhonov, 2004). A residue label includes the domain number (1–4), segment type (k, the linker-helix between S4 and S5; i, the inner helix S6; and o, the outer helix S5), and relative number of the residue in the segment. This allows the same labels to be applied to residues in matching positions of the sequence alignment of sodium channels from different organisms whose genuine residue numbers are different. The scheme also highlights the symmetric location of residues in different channel domains. The corresponding positions A1548V and A1648V from Pittendrigh et al. (1997) are indicated in brackets.

B. Aligned sequences of K_v1.2, Na_vAb and BgNa_v1-1 channels. Light gray and gray colors highlight, respectively, residues in the II/III and I/II domain interfaces that contribute to PyR1 and PyR2 or control ligand access to these receptors. Substitutions of these residues were tested experimentally, see (Du et al., 2015) and references therein. Underlined residues are mutations explored in this work.

Figure 2.

Effects of A^{3k10V} and A^{3p47V} mutations on the gating of BgNa_v1-1a channels. A. Voltage dependence of activation. B. Voltage dependence of inactivation. Voltage step protocols used to generate these curves are indicated above each panel.

Figure 3.

Effects of A^{3k10V} and A^{3p47V} mutations on the sensitivity of $BgNa_v1-1a$ channels to permethrin (PMT) and deltamethrin (DMT). A and B. Tail currents induced by PMT or DMT from $BgNa_v1-1a$ and mutant channels. C. Percentage of channel modification by 1.0 μM PMT or 1.0 μM DMT as determined by the method of Tatebayashi and Narahashi (1994). The number of oocytes for each mutant construct was > 5. Error bars indicate mean $\pm$ s.e.m. The asterisks indicate significant decrease from the $BgNa_v1-1a$ channel as determined by using one-way analysis of variance (ANOVA) with Scheffé's *post hoc* analysis, and significant values were set at $p < 0.05$.

Figure 4.

Effects of A^{3k10V} and A^{3p47V} mutations on the sensitivity of BgNa_v1-1a channels to cypermethrin (CPMT) and NRDC 157. A and B. Tail currents induced by CPMT or NRDC 157 from BgNa_v1-1a and mutant channels. C. Percentage of channel modification by 1.0 μM CPMT or 1.0 μM NRDC 157 as determined by the method of Tatebayashi and Narahashi (Tatebayashi and Narahashi, 1994). The number of oocytes for each mutant construct was > 5. Error bars indicate mean ± s.e.m. The asterisks indicate significant decrease from the BgNa_v1-1a channel as determined by using one-way ANOVA with Scheffé's *post hoc* analysis, and significant values were set at $p < 0.05$.

Figure 5. Effects of A^{3k10V} and A^{3p47V} mutations on the sensitivity of BgNa_v1-1a channels to DDT. A-C. Representative traces from BgNa_v1-1a, A^{3k10V} and A^{3p47V} sodium channels after incubation with DDT (100 μM). D. Percentages of channel inactivation inhibited by DDT (100 μM). The number of oocytes for each mutant construct was > 8. Error bars indicate mean ± s.e.m. The asterisks indicate significant differences ($p < 0.05$) in sensitivity of mutants versus wildtype to DDT as determined by using one-way ANOVA with Scheffé's *post hoc* analysis.

Figure 6.

Contacts of residues A^{3p47} and A^{3k10}. Cytoplasmic (A) and side (B) views of the open-state BgNav_v1-1a sodium channel model, which is identical to the mosquito sodium channel model of AaNav_v1-1 (Du et al., 2015). Roman numerals indicate domains. Residues A^{3p47} and A^{3k10} are space-filled and colored green. Residues that are in close contact with A^{3p47} and A^{3k10} are space-filled with gray carbons and hydrogens. C and D, close-up views at contacts of residues A^{3p47} and A^{3k10}.

Table 1
Effects of A^{3k10V} and A^{3p47V} mutations on the voltage-dependence of activation and inactivation.

Na ⁺ channel type	Activation		Inactivation		n
	V _{1/2} (mV)	k (mV)	V _{1/2} (mV)	k (mV)	
B ⁶ Na _v 1-1a	-30.2 ± 0.8	3.8 ± 0.2	-50.9 ± 0.4	4.5 ± 0.1	15
A ^{3k10V}	-15.2 ± 0.6 *	5.7 ± 0.2	-56.7 ± 0.4 *	5.5 ± 0.1	15
A ^{3p47V}	-17.3 ± 0.6 *	4.6 ± 0.2	-50.0 ± 0.4	5.2 ± 0.2	15

The values in the table represent the mean ± s.e.m. and n is the number of oocytes used.
* Significantly different from the wildtype using one-way ANOVA with Scheffé's *post hoc* analysis (p < 0.05).

Table 2

Effects of A^{3k10V} and A^{3p47V} mutations on the tail current decay of BgNa_v1-1a channels to deltamethrin (DMT) and cypermethrin (CPMT).

Na ⁺ channel type	Integral modified channels		n
	Deltamethrin	Cypermethrin	
BgNa _v 1-1a	457 ± 28	444 ± 45	8
A ^{3k10V}	296 ± 26 *	234 ± 39 *	7
A ^{3p47V}	250 ± 19 *	215 ± 20 *	7

The value in the table represent the mean ± s.e.m. and n is the number of oocytes used.

* Significantly different from the wildtype using one-way ANOVA with Scheffé's *post hoc* analysis ($p < 0.05$).