Published in final edited form as:

Insect Biochem Mol Biol. 2017 March ; 82: 1–10. doi:10.1016/j.ibmb.2017.01.007.

Mutations of two acidic residues at the cytoplasmic end of segment IIS6 of an insect sodium channel have distinct effects on pyrethroid resistance

Mengli Chen^{a,b}, Yuzhe Du^{b,1}, Yoshiko Nomura^b, Guonian Zhu^a, Boris S. Zhorov^{c,d}, and Ke Dong^{b,*}

^aInstitute of Pesticide and Environmental Toxicology, Zhejiang University, Hangzhou 310029, China

^bDepartment of Entomology, Genetics and Neuroscience Programs, Michigan State University, East Lansing, MI48824, USA

^cDepartment of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario L8N 3Z5, Canada

^dSechenov Institute of Evolutionary Physiology & Biochemistry, Russian Academy of Sciences, St. Petersburg 194223, Russia

Abstract

Mutations in sodium channels are known to confer knockdown resistance to pyrethroid insecticides, such as permethrin and cypermethrin, in various agricultural pests and disease vectors. Double mutations, D³ⁱ²⁸V and E³ⁱ³²G, were detected in cypermethrin-resistant *Helicoverpa armigera* and *Heliothis virescens* populations. However, the role of the two mutations in pyrethroid resistance remains unclear. In this study, we introduced the mutations into the cockroach sodium channel, BgNa_v1-1a, and examined their effects on channel gating and pyrethroid sensitivity in *Xenopus* oocytes. D³ⁱ²⁸V alone and the double mutation, D³ⁱ²⁸V/E³ⁱ³²G, shifted the voltage dependence of activation in the depolarizing direction by 15 mV and 20 mV, respectively, whereas E³ⁱ³²G had no significant effect. D³ⁱ²⁸V reduced the amplitude of tail currents induced by permethrin and NRDC 157 (Type I pyrethroids) and deltamethrin and cypermethrin (Type II pyrethroids), whereas E³ⁱ³²G alone had no effect. Intriguingly, the amplitude of Type II pyrethroid-induced tail current from D³ⁱ²⁸V/E³ⁱ³²G channels was similar to that of BgNa_v1-1a channels, but the decay of the tail currents was accelerated. Such effects were not observed for Type I pyrethroid-induced tail currents. Computational analysis based on the model of dual pyrethroid receptors on insect sodium channels predicted D³ⁱ²⁸V and E³ⁱ³²G exert their effects on channel gating and pyrethroid action via allosteric mechanisms. Our results not only illustrate the distinct effect of the D³ⁱ²⁸V/E³ⁱ³²G double mutations on Type I vs. Type II pyrethroids, but also reinforce the concept that accelerated decay of tail currents can be an effective mechanism of pyrethroid resistance to Type II pyrethroids.

*Corresponding author: dongk@cns.msu.edu (K. Dong).

¹Current address: USDA-ARS, Biological Control of Pest Research Unit, 59 Lee Road, Stoneville, MS 38776, USA.

Keywords

Sodium channel; Knockdown resistance; Type I pyrethroids; Type II pyrethroids; Pyrethroid receptor

1. Introduction

Voltage-gated sodium channels are essential for the generation and propagation of action potentials in almost all excitable cells (Catterall, 2012). The processes of sodium channel activation and inactivation play critical roles in regulating membrane excitability. Sodium channels are the major target for a variety of naturally occurring neurotoxins and synthetic compounds, including pyrethroid insecticides (Catterall et al., 2007; Narahashi, 1996). Pyrethroids preferably interact with open insect sodium channels, causing prolonged opening of the channels by inhibiting their inactivation and deactivation (Bloomquist, 1996; Bloomquist and Soderlund, 1988; Narahashi, 1988, 2000; Narahashi et al., 1992; Silver et al., 2014; Vijverberg and van den Bercken, 1982; Vijverberg et al., 1982).

Because of their fast action, potent insecticidal activity and low mammalian toxicity, pyrethroids are widely used for controlling arthropod pests and human disease vectors. However, intensive use of pyrethroids has led to the selection of resistance in many insect species around the world. One of major mechanisms of pyrethroid resistance, which is known as knockdown resistance (kdr), results from reduced neuronal sensitivity to DDT and pyrethroids (Soderlund and Bloomquist, 1990). Many kdr mutations have been found in the sodium channel genes, and sodium channels carrying kdr mutations, when expressed in *Xenopus* oocytes, exhibit reduced sensitivity to pyrethroids (Davies et al., 2007; Dong, 2007; Soderlund, 2005). Experimental evidence suggests that some kdr mutations alter pyrethroid binding to the sodium channels, whereas other mutations affect pyrethroid action by modifying the gating properties of sodium channels (Du et al., 2006). Overall, identification of these kdr mutations has greatly improved our understanding of the molecular mechanism of action of pyrethroids on sodium channels.

Pyrethroids are classified as Type I and Type II based on their chemical structures and poisoning symptoms. Structurally, Type I and Type II pyrethroids differ mainly in the absence (Type I) or the presence (Type II) of an α -cyano group at the phenylbenzyl alcohol position. Type I pyrethroids induce repetitive discharges with a single electrical stimulus, whereas Type II pyrethroids cause membrane depolarization accompanied by suppression of action potentials following multiple electrical stimuli (Gammon et al., 1981; Lund and Narahashi, 1981b). In addition, although both Type I and Type II pyrethroids prolong sodium channel opening by inhibiting inactivation and deactivation, Type II pyrethroids inhibit the deactivation of sodium channel to a greater extent than Type I pyrethroids. Consequently, the decay of tail currents induced by Type II pyrethroids is much slower than those induced by Type I pyrethroids (Lund and Narahashi, 1981a; Narahashi, 1988; Vijverberg et al., 1982).

McCaffery et al. (1997) reported several cotton bollworm (*Helicoverpa armigera*) and tobacco budworm (*Heliothis virescens*) populations from the United States and China, which

exhibited high levels of nerve insensitivity to cypermethrin, a Type II pyrethroid. Subsequently, double mutations, D1561V and E1565G, (also known as D³ⁱ²⁸V and E³ⁱ³²G according to the nomenclature of ion channels for their positions at the intracellular end of the segment 6 of domain III) were identified in the sodium channels from these pyrethroid-resistant strains (Head et al., 1998). However, whether or not these two mutations confer sodium channel resistance to pyrethroids remains to be determined. Functional expression of sodium channels from Lepidopteran species has not been established in *Xenopus* oocytes. The cockroach sodium channel expression system has been reliable to confirm the role of sodium channel mutations from other insect and arachnid species in pyrethroid resistance (Du et al., 2013-; Hu et al., 2011; Liu et al., 2006). Therefore, we introduced D³ⁱ²⁸V, E³ⁱ³²G and D³ⁱ²⁸V/E³ⁱ³²G mutations into the previously characterized German cockroach sodium channel, BgNa_v1-1a, and examined the pyrethroid sensitivity of the resulting mutant channels expressed in *Xenopus* oocytes. Our study reveals distinct roles of the double mutations in cockroach sodium channel resistance to Type I and Type II pyrethroids and provides new insights into the molecular interactions between sodium channels with Type I vs. Type II pyrethroids.

2. Materials and methods

2.1. Site-directed mutagenesis

A pyrethroid-sensitive sodium channel variant from the German cockroach, BgNa_v1-1a, was modified to generate mutants D³ⁱ²⁸V, E³ⁱ³²G and a double mutant D³ⁱ²⁸V/E³ⁱ³²G (Fig. 1). Site-directed mutagenesis was performed by PCR on the entire clone 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

Ovaries from oocyte-positive female *Xenopus laevis* were purchased from Xenopus 1 (Dexter, MI). The procedures for oocyte preparation, cRNA synthesis and injection were identical to those described previously (Tan et al., 2002a). 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, the BgNa_v1-1a cRNA was co-injected into oocytes with *Drosophila melanogaster* tipE cRNA (1:1 M ratio), which enhances the expression of insect sodium channels (Feng et al., 1995; Warmke et al., 1997), but does not alter the sensitivity of cockroach sodium channels to pyrethroids (Data not shown).

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 current. Peak conductance values were normalized to the maximal peak conductance ($G_{\max}$) and fitted with a two-state Boltzmann equation of the form:

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

where 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-millisecond 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 ms. 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:

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

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

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

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 currents 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. The Dose-response curves were fitted to the Hill Equation: $M = M_{\max} / \{ 1 + (K_d / [\text{pyrethroids}])^n \}$, where

[pyrethroids] and K_d represent the concentration of pyrethroids and the concentration to produce the half maximal effect, respectively, n represents the Hill coefficient. The $M_{\max}$ is the maximal percentage of sodium channels modified.

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

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

where $I_{\text{tail, integ}}$ 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.

2.6. Molecular modeling

We use a residue labeling scheme, which is universal for P-loop channels (Zhorov and Tikhonov, 2004), including insect channels (Du et al., 2013). According to this scheme, a residue label includes the repeat number, letter k, o, or i, which designates, respectively, the linker helix L45, the outer helix S5, or the inner helix S6, and the relative number of the residue in the respective helix (Fig. 1B). Previous modeling of the PyR1 and PyR2 receptors did not include D^{3i28} or E^{3i32} (Du et al., 2013, 2015), therefore we upgraded our open and closed models of the BgNa_v1-1a channel by using, as templates, the X-ray structures of the closed chimeric sodium channel Na_vAb/Na_v1.7 (Ahuja et al., 2015) and open chimeric potassium channel K_v1.2/K_v2.1 (Long et al., 2007). In these structures, the S6 helices include residues i32 and linker-helices L45 are resolved. The sequence alignment of relevant segments in the Na_vAb, K_v1.2 and BgNa_v1-1a channels is shown in Fig. 1B. We further optimized state-dependent contacts of residues D^{3i28} and E^{3i32} in the BgNa_v1-1a channel and its $D^{3i28}V$ and $D^{3i28}V/E^{3i32}G$ mutants by Monte Carlo-minimizing energy of the cytoplasmic part of repeat III as described elsewhere (Du et al., 2015).

3. Results

3.1. Mutations $D^{3i28}V$ and $D^{3i28}V/E^{3i32}G$ alter the gating properties of the BgNa_v1-1a channel

We used a pyrethroid-sensitive cockroach sodium channel variant, BgNa_v1-1a, to generate three mutant channels carrying the $D^{3i28}V$, $E^{3i32}G$ or $D^{3i28}V/E^{3i32}G$ mutations, respectively. Expression of these mutant channels in *Xenopus* oocytes generated sufficient currents for examination of their gating properties and sensitivity to pyrethroids. The $E^{3i32}G$ substitution did not significantly alter the voltage dependence of either activation or inactivation. However, the $D^{3i28}V$ and $D^{3i28}V/E^{3i32}G$ substitutions shifted the voltage dependence of activation in the depolarizing direction by 15 mV and 20 mV, respectively, but had no effect on the reversal potential. None of the mutations had any effect on the voltage dependence of channel inactivation (Table 1 and Fig. 2).

3.2. Mutation D³ⁱ²⁸V, but not E³ⁱ³²G, conferred BgNa_v1-1a channel resistance to both Type I and Type II pyrethroids

Because pyrethroids preferably bind to open sodium channels, we used multiple short depolarizations to detect tail currents induced by pyrethroids (Vais, 2000) to examine the effects of a Type I pyrethroid, permethrin (PMT), and a Type II pyrethroid, deltamethrin (DMT), on the mutant channels in comparison with the wildtype channel BgNa_v1-1a. No tail currents were detected in the absence of pyrethroids in BgNa_v1-1a and mutant channels using this protocol (Fig. 3A and B). Both PMT and DMT induced significant tail currents in BgNa_v1-1a channel (Fig. 3A and B). Tail currents induced by PMT decayed quickly, which returned to baseline within 2 s, whereas tail currents induced by DMT decayed slowly, over several minutes. The amplitudes of PMT- and DMT-induced tail currents increased in a dose-dependent manner (Fig. 3A and B). E³ⁱ³²G did not reduce the amplitudes of tail currents induced by either PMT or DMT (Fig. 3A and B). In contrast, the amplitudes of pyrethroid-induced tail currents were reduced in D³ⁱ²⁸V channels. For D³ⁱ²⁸V channels, the percentage of channel modification by 1.0 μM PMT or DMT using the method by Tatebayashi and Narahashi (1994) was less than 25%, which was much less than the 95% modification seen in wildtype channel, a reduction of 5- or 4- folds, respectively (Fig. 3C).

3.3. Double mutation D³ⁱ²⁸V/E³ⁱ³²G accelerated the decay of Type II pyrethroid-induced tail currents

The percentage of channel modification by PMT of the D³ⁱ²⁸V/E³ⁱ³²G mutant channel was comparable to that of the D³ⁱ²⁸V channel (Fig. 3C), indicating that the E³ⁱ³²G mutation had no additional effect on the action of PMT. As for the effect of DMT, unlike the D³ⁱ²⁸V channel, the amplitude of DMT-induced tail currents was not reduced in D³ⁱ²⁸V/E³ⁱ³²G double mutant channel, but was instead comparable to that in wildtype channel (Fig. 3C). Interestingly, the DMT-induced tail currents from D³ⁱ²⁸V/E³ⁱ³²G channel decayed faster than those from the wildtype (Fig. 3B). The unique effects of the D³ⁱ²⁸V/E³ⁱ³²G double mutations on tail current decay, caused us to consider both the amplitude and the rate of decay in further evaluation of the potency of DMT on wildtype and mutant channels. Accordingly, we used the method of Usherwood et al. (2007) to determine the level of integral channel modification. The D³ⁱ²⁸V/E³ⁱ³²G double mutations reduced integral channel modification by 1.6 fold compared with the wildtype channel (Table 2), the D³ⁱ²⁸V mutation alone drastically reduced integral channel modification by about 10 fold, and the E³ⁱ³²G mutation had no effect.

Since the D³ⁱ²⁸V/E³ⁱ³²G mutations were originally identified in cypermethrin (CPMT)-resistant populations, we also evaluated the action of CPMT on the three mutant channels. CPMT, a Type II pyrethroid, differs structurally from PMT only by the presence of the α-cyano group. As shown in Fig. 4, mutation D³ⁱ²⁸V reduced channel sensitivity to CPMT, whereas E³ⁱ³²G had no effect. Although the amplitude of tail currents induced by CPMT from D³ⁱ²⁸V/E³ⁱ³²G channel was comparable to those from the wildtype and E³ⁱ³²G channels, the integral channel modification by CPMT of D³ⁱ²⁸V/E³ⁱ³²G channel was reduced by 2.5-fold compared with the wildtype and E³ⁱ³²G channels (Fig. 4 and Table 2). Therefore, the effects of the three mutations on the action of CPMT versus DMT were similar.

We also examined the effects of the three mutations on the action of NRDC 157, which is a DMT analog lacking the α -cyano group (i.e., a Type I pyrethroid). The results were similar to those of PMT (Fig. 4A and C). The D³ⁱ²⁸V and D³ⁱ²⁸V/E³ⁱ³²G channels exhibited similar levels of resistance to NRDC 157, whereas E³ⁱ³²G channel was as sensitive to NRDC 157 as wildtype channel (Fig. 4A and C). We could not quantify the integral channel modification by PMT and NRDC 157 due to the extremely rapid decay of tail currents induced by the Type I pyrethroids.

3.4. State-dependent inter-helical contacts of residues i28 and i32 in the BgNa_v1-1a channel and its mutants

In our latest model of pyrethroid receptors PyR1 and PyR2 (Du et al., 2015) residue D³ⁱ²⁸ is too far from the bound pyrethroids and residue E³ⁱ³² (whose analog is absent in the X-ray structure which was used to build the model) would be even farther. Therefore, according to our models, mutations D³ⁱ²⁸V and E³ⁱ³²G would affect binding of pyrethroids to PyR1 and/or PyR2 by allosteric mechanisms, e.g. by changing the channel gating. In the closed wildtype channel model, E³ⁱ³² is not involved in any intersegment contacts (Fig. 5A). D³ⁱ²⁸ approaches S^{3o5}, but does not form favorable contacts with it (Fig. 5A). Instead, the S^{3o5} hydroxyl donates an H-bond to the backbone carbonyl of Q^{3o1} and this H-bond induces a kink between the linker-helix IIL45 and outer-helix IIS5. Proline P^{3o4}, which lacks the backbone NH group, cannot donate an H-bond to the Q^{3o1} backbone. This makes the S^{3o5}—Q^{3o1} H-bond particularly strong and H-bond S^{3o5}—D³ⁱ²⁸ unlikely. In our model the carboxylate group of D³ⁱ²⁸ approaches the methylene group of S^{3o5} (Fig. 5A) and this unfavorable intersegment contact destabilizes the closed-state energy.

In the open-state model of wildtype BgNa_v1-1a, D³ⁱ²⁸ and E³ⁱ³² accept intersegment H-bonds from Q^{3o1} and N^{3k9} in the linker-helix IIL45 (Fig. 5B). Mutation D³ⁱ²⁸V would stabilize the closed channel by forming a favorable contact between V³ⁱ²⁸ and the methylene group of S^{3o5} and destabilize the open state by eliminating the H-bond with Q^{3o1}. This would make the channel harder to open in agreement with the depolarizing shift of activation in the D³ⁱ²⁸V mutant (Fig. 2A).

In the double mutant D³ⁱ²⁸V/E³ⁱ³²G both H-bonds between the C-terminal part of IIS6 and the linker-helix IIL45 are lost (Fig. 5B, lower panel). On one hand, this would further destabilize the open channel energy in agreement with the strong depolarizing shift seen in the voltage of activation (Fig. 2A). On the other hand, in the absence of the intersegment open-state H-bonds, the C-terminal half of IIS6 would become more mobile and may shift to adjust to the PyR1 bound ligand in the IIL45-IIS6 interface. A smaller shift is expected for the N-terminal half of IIS6, which is engaged in strong inter-helical contacts with IIS5 and IIP1 helices.

In our open-state model of an insect sodium channel, Type II pyrethroid deltamethrin binds deeper (closer to the cytoplasm) than Type I pyrethroid permethrin (cf. Fig. 3B and C in Du et al. (2013)). In our model of PyR1-bound deltamethrin (Du et al., 2015) the ligand reaches residue I³ⁱ²³, which is just five positions above D³ⁱ²⁸. We suggest that in the double mutant the conformational shift of the IIS6 C-terminal part would strengthen its contact with Type II, but not Type I pyrethroids. This can explain the paradoxical fact that the double mutation

D³ⁱ²⁸V/E³ⁱ³²G rescues the negative impact of the point mutation D³ⁱ²⁸V on Type II, but not Type I pyrethroids (Figs. 3C and 4C).

4. Discussion

In this study, we examined the effects of two sodium channel mutations, D³ⁱ²⁸V and E³ⁱ³²G, which were detected in cypermethrin-resistant *H. armigera*/*H. virescens* populations, on pyrethroid sensitivity of a cockroach sodium channel. Our results not only confirmed the role of the double mutations in cypermethrin resistance, but also revealed that accelerating the decay of Type II-induced tail currents by *kdr* mutations could be a distinct mechanism by which insects confer pyrethroid resistance to Type II pyrethroids.

In voltage-clamp experiments, the amplitude of pyrethroid-induced tail currents represents the peak of sodium currents through the pyrethroid-modified open channels upon repolarization, whereas the decay of tail currents reflects the closing of the pyrethroid-modified channels. One major difference in the actions of Type I and Type II pyrethroids is the extent to which pyrethroids keep sodium channels in the open state. In general, sodium channels modified by Type I pyrethroids return to the closed state very rapidly, within seconds, upon repolarization, whereas Type II pyrethroids induce tail currents that decay very slowly, often minutes after repolarization (Narahashi, 1988). Most of the examined *kdr* sodium channel mutations greatly reduce the amplitude of tail currents induced by both Type I and Type II pyrethroids (Du et al., 2013, 2015; Lee et al., 1999; Tan et al., 2002a, 2005; Vais et al., 2001; Warmke et al., 1997). However, recently we found that two pyrethroid resistance-conferring alanine to valine substitutions in the pore helix IIIIP1 and linker-helix IIIL45 of *Drosophila melanogaster* do not reduce the amplitude of tail currents induced by Type II pyrethroids, but accelerate the decay of the tail currents (Chen et al., 2016).

In this study, we presented a more complex scenario which involves double mutations exerting distinct effects on the amplitude and decay of tail currents induced by Type I and Type II pyrethroids. High levels of pyrethroid resistance could be conferred by the apparently additive effects of double or triple mutations (Hirata et al., 2014; Vais et al., 2003). In some cases, though, where multiple mutations are involved, it was found that there are primary and secondary mutations. Often, secondary mutations alone have no effect on the action of pyrethroids, but in the presence of a primary mutation, these secondary mutations could enhance the level of resistance by further reducing the amplitude of tail currents (Liu and Pridgeon, 2002; Tan et al., 2002b; Wang et al., 2015). In the case of D³ⁱ²⁸V and E³ⁱ³²G, it seems that D³ⁱ²⁸V is a primary mutation, which alone can confer resistance to pyrethroids, and E³ⁱ³²G is a secondary mutation because it alone does not alter the action of either Type I or Type II pyrethroids. However, the role of the E³ⁱ³²G mutation in pyrethroid resistance is unprecedented. First, E³ⁱ³²G unexpectedly abolishes (i.e., not enhances) the D³ⁱ²⁸V-mediated reduction of the amplitude of Type II-induced tail currents, but had no effect on Type I-induced tail currents. Secondly, E³ⁱ³²G accelerates the decay of Type II-induced tail currents when combined with the primary D³ⁱ²⁸V mutation. Consequently, the D³ⁱ²⁸V/E³ⁱ³²G double mutations confer resistance to Type I pyrethroids including permethrin by reducing the amplitude of Type I-induced tail currents, and to Type

II pyrethroids including cypermethrin not by decreasing amplitude, but by accelerating the decay of Type II-induced tail currents.

Double mutations, D³ⁱ²⁸V and E³ⁱ³²G, were detected in cypermethrin-resistant *H. armigera* and *H. virescens* strains (Head et al., 1998). The D³ⁱ²⁸V mutation shifted the voltage dependence of activation in the depolarizing direction, and the E³ⁱ³²G mutation further enhanced the gating modification caused by the D³ⁱ²⁸V mutation. The drastic depolarizing shift in gating could lead to decreased neuronal excitability in individuals carrying the double mutations. Besides D³ⁱ²⁸V and E³ⁱ³²G, different pyrethroid resistance-associated mutations, L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in IIS6 and V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M in IS6, were found in *H. virescens* species in other populations in various regions in the US (Park et al., 1997a, Park and Taylor, 1997b; Park et al., 2000). Both L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in IIS6 and V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M mutations also modify the voltage dependence of activation, but less so than D³ⁱ²⁸V/E³ⁱ³²G. While D³ⁱ²⁸V/E³ⁱ³²G cause a 15–20 mV depolarizing shift in the voltage dependence of activation, L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in IIS6 and V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M cause ca. 4 mV and 7 mV depolarizing shifts, respectively. It was predicted that V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M was associated with fitness costs and later was replaced with L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in *H. virescens* populations (Zhao et al., 2000). In 2010 in Texas, both L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in IIS6 and V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M/A/G in IS6 appear to have prevailed in *H. zea* species, but D³ⁱ²⁸V and E³ⁱ³²G were not detected (Hopkins and Piertrantonio, 2010). D³ⁱ²⁸V/E³ⁱ³²G may impose a more serious fitness cost than L²ⁱ¹⁶ⁱ¹⁰ⁱ¹⁴H in IIS6 and V¹ⁱ¹⁹⁽⁴ⁱ¹⁰⁾M/A/G due to more drastic effects on sodium channel functions.

Voltage sensing helices (segments S4) in each domain of P-loop voltage-gated channels are coupled with the activation gate formed by the S6 helices. Complex conformational changes in the voltage-sensing domains in response to membrane potential changes trigger opening of the pore region via the S4-S5 linker. (Chanda and Bezanilla, 2002; Gosselin-Badaroudine et al., 2015). It does not seem surprising that residues at the intracellular end of IIS6, such as D³ⁱ²⁸ and E³ⁱ³², are involved in the channel activation. Mutation D³ⁱ²⁸V eliminates a negative charge at the intracellular end of IIS6 and thus could impair the coupling of the voltage sensor outward movement with the activation gate opening. In view of our model, the fact that mutation E³ⁱ³²G enhances the D³ⁱ²⁸V-induced depolarizing shift in the voltage dependence of activation (Fig. 2A) can be explained by elimination of two IIS6-IIIIL45 H-bonds that stabilize the open channel.

In our BgNa_v1-1a models, D³ⁱ²⁸ and E³ⁱ³² are beyond the pyrethroid receptors, implying allosteric effects of the mutations. D³ⁱ²⁸ has unfavorable closed-state contacts, but forms an open-state H-bond with IIIIL45. Mutation D³ⁱ²⁸V is predicted to stabilize the closed and destabilize the open state, in agreement with the observed depolarizing activation shift. E³ⁱ³² lacks closed-state intersegment contacts, but forms an open-state H-bond with IIIIL45. The loss of the two H-bonds in the double mutant would increase the open-state mobility of the C-terminal part of IIS6. In our published models Type II pyrethroids bind to IIS6 deeper than Type I pyrethroids. A shift of IIS6 may explain why E³ⁱ³²G negates the impact of D³ⁱ²⁸V on the tail current induced by Type II pyrethroids, but not Type I pyrethroids.

Acknowledgments

We thank Dr. Kris Silver for critical review of this manuscript. Computations were performed using the facilities of the Shared Hierarchical Academic Research Computing Network (SHARCNET, www.sharcnet.ca). This study was supported by the National Institutes of Health National Institute of General Medical Sciences (Grant GM057440 to KD and BSZ) and the Natural Sciences and Engineering Research Council of Canada (Grant RGPIN-2014-04894 to BSZ). Mengli Chen is supported by the China Scholarship Council.

References

- Ahuja S, Mukund S, Deng L, Khakh K, Chang E, Ho H, Shriver S, Young C, Lin S, Johnson JP Jr, Wu P, Li J, Coons M, Tam C, Brillantes B, Sampang H, Mortara K, Bowman KK, Clark KR, Estevez A, Xie Z, Verschoof H, Grimwood M, Dehnhardt C, Andrez JC, Focken T, Sutherlin DP, Safina BS, Starovasnik MA, Ortwine DF, Franke Y, Cohen CJ, Hackos DH, Koth CM, Payandeh J. Structural basis of Nav1.7 inhibition by an isoform-selective small-molecule antagonist. *Science*. 2015; 350:aac5464. [PubMed: 26680203]
- Bloomquist JR. Ion channels as targets for insecticides. *Annu Rev Entomol*. 1996; 41:163–190. [PubMed: 8546445]
- Bloomquist JR, Soderlund DM. Pyrethroid insecticides and DDT modify alkaloid-dependent sodium channel activation and its enhancement by sea anemone toxin. *Mol Pharmacol*. 1988; 33:543–550. [PubMed: 2452970]
- Catterall W. Voltage-gated sodium channels at 60: structure, function and pathophysiology. *J Physiol*. 2012; 590:2577–2589. [PubMed: 22473783]
- Catterall WA, Cestele S, Yarov-Yarovoy V, Yu FH, Konoki K, Scheuer T. Voltage-gated ion channels and gating modifier toxins. *Toxicon*. 2007; 49:124–141. [PubMed: 17239913]
- Chanda B, Bezanilla F. Tracking voltage-dependent conformational changes in skeletal muscle sodium channel during activation. *J Gen Physiol*. 2002; 120:629–645. [PubMed: 12407076]
- Chen M, Du Y, Nomura Y, Zhu G, Zhorov BS, Dong K. Alanine to valine substitutions in the pore helix IIIP1 and linker-helix IIIL45 confer cockroach sodium channel resistance to DDT and pyrethroids. *Neurotoxicology*. 2016 In press.
- Davies T, Field L, Usherwood P, Williamson M. A comparative study of voltage-gated sodium channels in the Insecta: implications of pyrethroid resistance in Anopheline and other Neopteran species. *Insect Mol Biol*. 2007; 16:361–375. [PubMed: 17433068]
- Dong K. Insect sodium channels and insecticide resistance. *Invert Neurosci*. 2007; 7:17–30. [PubMed: 17206406]
- Du Y, Liu Z, Nomura Y, Khambay B, Dong K. An alanine in segment 3 of domain III (IIIS) of the cockroach sodium channel contributes to the low pyrethroid sensitivity of an alternative splice variant. *Insect Biochem Molec Biol*. 2006; 36:161–168. [PubMed: 16431283]
- Du Y, Nomura Y, Satar G, Hu Z, Nauen R, He SY, Zhorov BS, Dong K. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. *Proc Natl Acad Sci U S A*. 2013; 110:11785–11790. [PubMed: 23821746]
- Du Y, Nomura Y, Zhorov BS, Dong K. Rotational symmetry of two pyrethroid receptor sites in the mosquito sodium channel. *Mol Pharmacol*. 2015; 88:273–280. [PubMed: 25972447]
- Feng G, Deak Pt, Chopra M, Hall LM. Cloning and functional analysis of tipE, a novel membrane protein that enhances drosophila *para* sodium channel function. *Cell*. 1995; 82:1001–1011. [PubMed: 7553842]
- Gammon DW, Brown MA, Casida JE. Two classes of pyrethroid action in the cockroach. *Pestic Biochem Physiol*. 1981; 15:181–191.
- Gosselin-Badaroudine P, Moreau A, Delemotte L, Cens T, Collet C, Rousset M, Charnet P, Klein ML, Chahine M. Characterization of the honeybee AmNaV1 channel and tools to assess the toxicity of insecticides. *Sci Rep*. 2015; 5:12475. [PubMed: 26202396]
- Head D, McCaffery A, Callaghan A. Novel mutations in the para-homologous sodium channel gene associated with phenotypic expression of nerve insensitivity resistance to pyrethroids in *Heliothis lepidoptera*. *Insect Molec Biol*. 1998; 7:191–196. [PubMed: 9535164]

- Hirata K, Komagata O, Itokawa K, Yamamoto A, Tomita T, Kasai S. A single crossing-over event in voltage-sensitive Na⁺ channel genes may cause critical failure of dengue mosquito control by insecticides. *PLoS Negl Trop Dis*. 2014; 8:e3085. [PubMed: 25166902]
- Hopkins BW, Piertrantonio PV. The *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) voltage-gated sodium channel and mutations associated with pyrethroid resistance in field-collected adult males. *Insect Biochem Molec Biol*. 2010; 40:385–393. [PubMed: 20362057]
- Hu Z, Du Y, Nomura Y, Dong K. A sodium channel mutation identified in *Aedes aegypti* selectively reduces cockroach sodium channel sensitivity to type I, but not type II pyrethroids. *Insect Biochem Molec Biol*. 2011; 41:9–13. [PubMed: 20869441]
- Lee A, Wong ST, Gallagher D, Li B, Storm DR, Scheuer T, Catterall WA. Ca²⁺/calmodulin binds to and modulates P/Q-type calcium channels. *Nature*. 1999; 399:155–159. [PubMed: 10335845]
- Liu A, Williamson MS, Lansdell SJ, Han Z, Denholm I, Millar NS. A nicotinic acetylcholine receptor mutation (Y151S) causes reduced agonist potency to a range of neonicotinoid insecticides. *J Neurochem*. 2006; 99:1273–1281. [PubMed: 16981889]
- Liu N, Pridgeon JW. Metabolic detoxication and the *kdr* mutation in pyrethroid resistant house flies, *Musca domestica* (L. Pestic. Biochem Physiol. 2002; 73:157–163.
- Long SB, Tao X, Campbell EB, MacKinnon R. Atomic structure of a voltage-dependent K⁺ channel in a lipid membrane-like environment. *Nature*. 2007; 450:376–382. [PubMed: 18004376]
- Lund AE, Narahashi T. Kinetics of sodium channel modification by the insecticide tetramethrin in squid axon membranes. *J Pharmacol Exp Ther*. 1981a; 219:464–473. [PubMed: 6270310]
- Lund AE, Narahashi T. Modification of sodium channel kinetics by the insecticide tetramethrin in crayfish giant axons. *Neurotoxic. 1981b; 2:213–229.*
- McCaffery AR, Head DJ, Jianguo T, Dubbeldam AA, Subra-maniam VR, Callaghan A. Nerve insensitivity resistance to pyrethroids in *Heliothis* Lepidoptera. *Pestic Sci*. 1997; 51:315–320.
- Narahashi, T. Neurotox 88: Molecular Basis of Drug and Pesticide Action. Elsevier; New York: 1988. Molecular and cellular approaches to neurotoxicology: past, present and future.
- Narahashi T. Neuronal ion channels as the target sites of insecticides. *Pharmacol Toxicol*. 1996; 78:1–14. [PubMed: 8821391]
- Narahashi T. Neuroreceptors and ion channels as the basis for drug action: past, present, and future. *J Pharmacol Exp Ther*. 2000; 294:1–26. [PubMed: 10871290]
- Narahashi T, Frey JM, Ginsburg KS, Roy ML. Sodium and GABA-activated channels as the targets of pyrethroids and cyclodienes. *Toxicol Lett*. 1992; 64–65(Spec No):429–436.
- Park Y, Taylor MF, Feyereisen R. A valine421 to methionine mutation in IS6 of the hscp voltage-gated sodium channel associated with pyrethroid resistance in *Heliothis virescens* F. *Biochem Biophys Res Commun*. 1997a; 239:688–691. [PubMed: 9367829]
- Park Y, Taylor MF. A novel mutation L1029H in sodium channel gene hscp associated with pyrethroid resistance for *Heliothis virescens* (Lepidoptera: Noctuidae). *Insect Biochem Mol Biol*. 1997b; 27:9–13. [PubMed: 9061924]
- Park YS, Lee DW, Taylor MFJ, Holloway JW, Ottea JA, Adams ME, Feyereisen R. A mutation Leu1029 to His in *Heliothis virescens* F-hscp sodium channel gene associated with a nerve-insensitivity mechanism of resistance to pyrethroid insecticides. *Pestic Biochem Phys*. 2000; 66:1–8.
- Silver KS, Du Y, Nomura Y, Oliveira EE, Salgado VL, Zhorov BS, Dong K. Voltage-gated sodium channels as insecticide target sites. *Pestic Biochem Physiol*. 2014 In Press.
- Soderlund, DM. Sodium channels. In: Gilbert, LI, Iatrou, K., Gill, SS., editors. *Comprehensive Molecular Insect Science*. Elsevier; Amsterdam: 2005. p. 1-24.
- Soderlund, DM., Bloomquist, JR. Molecular mechanisms of insecticide resistance. In: Roush, RT., Tabashnik, BE., editors. *Pesticide Resistance in Arthropods*. Chapman and Hall; New York and London: 1990. p. 58
- Tan J, Liu Z, Nomura Y, Goldin AL, Dong K. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J Neurosci*. 2002a; 22:5300–5309. [PubMed: 12097481]

- Tan J, Liu Z, Tsai TD, Valles SM, Goldin AL, Dong K. Novel sodium channel gene mutations in *Blattella germanica* reduce the sensitivity of expressed channels to deltamethrin. *Insect Biochem Molec Biol.* 2002b; 32:445–454. [PubMed: 11886779]
- Tan J, Liu Z, Wang R, Huang ZY, Chen AC, Gurevitz M, Dong K. Identification of amino acid residues in the insect sodium channel critical for pyrethroid binding. *Mol Pharmacol.* 2005; 67:513–522. [PubMed: 15525757]
- Tatebayashi H, Narahashi T. Differential mechanism of action of the pyrethroid tetramethrin on tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. *J Pharmacol Exp Ther.* 1994; 270:595–603. [PubMed: 8071852]
- Usherwood PNR, Davies TGE, Mellor IR, O'Reilly AO, Peng F, Vais H, Khambay BPS, Field LM, Williamson MS. Mutations in DIIS5 and the DIIS4-S5 linker of *Drosophila melanogaster* sodium channel define binding domains for pyrethroids and DDT. *FEBS Letters.* 2007; 581(28):5485–5492. [PubMed: 17991435]
- Vais H, Atkinson S, Eldursi N, Devonshire AL, Williamson MS, Usherwood PN. A single amino acid change makes a rat neuronal sodium channel highly sensitive to pyrethroid insecticides. *FEBS Lett.* 2000a; 470:135–138. [PubMed: 10734222]
- Vais H, Atkinson S, Pluteanu F, Goodson SJ, Devonshire AL, Williamson MS, Usherwood PNR. Mutations of the *para* sodium channel of *Drosophila melanogaster* identify putative binding sites for pyrethroids. *Mol Pharmacol.* 2003; 64:914–922. [PubMed: 14500748]
- Vais H, Williamson MS, Devonshire AL, Usherwood PNR. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. *Pest Manag Sci.* 2001; 57:877–888. [PubMed: 11695180]
- Vais H, Williamson MS, Goodson SJ, Devonshire AL, Warmke JW, Usherwood PN, Cohen CJ. Activation of *Drosophila* sodium channels promotes modification by deltamethrin. Reductions in affinity caused by knockdown resistance mutations. *J Gen Physiol.* 2000b; 115:305–318. [PubMed: 10694259]
- Vijverberg H, van der Zalm JM, van der Bercken J. Similar mode of action of pyrethroids and DDT on sodium channel gating in myelinated nerves. *Nature.* 1982; 295:601–603. [PubMed: 6276777]
- Vijverberg HP, van den Bercken J. Annotation. Action of pyrethroid insecticides on the vertebrate nervous system. *Neuropathol Appl Neurobiol.* 1982; 8:421–440. [PubMed: 7162556]
- Wang L, Nomura Y, Du Y, Liu N, Zhorov BS, Dong K. A mutation in the intracellular loop III/IV of mosquito sodium channel synergizes the effect of mutations in helix IIS6 on pyrethroid resistance. *Mol Pharmacol.* 2015; 87:421–429. [PubMed: 25523031]
- Warmke J, Reenan RA, Wang P, Qian S, Arena JP, Wang J, Wunderler D, Liu K, Kaczorowski GJ, Van der Ploeg LH, Ganetzky B, Cohen CJ. Functional expression of *Drosophila para* sodium channels. Modulation by the membrane protein TipE and toxin pharmacology. *J Gen Physiol.* 1997; 110:119–133. [PubMed: 9236205]
- Zhao Y, Park Y, Adams ME. Functional and evolutionary consequences of pyrethroid resistance mutations in S6 transmembrane segments of a voltage-gated sodium channel. *Biochem Biophys Res Comm.* 2000; 278:516–521. [PubMed: 11095943]
- Zhorov BS, Tikhonov DB. Potassium, sodium, calcium and glutamate-gated channels: pore architecture and ligand action. *J Neurochem.* 2004; 88:782–799. [PubMed: 14756799]

Fig. 1.
A. Sodium channel topology indicating two mutations from *Helicoverpa armigera* and *Heliothis virescens* that were resistant to cypermethrin (Head et al., 1998). The positions of D³ⁱ²⁸V and E³ⁱ³²G are indicated in brackets. We use a residue-labeling scheme that is universal for P-loop channels (Du et al., 2013; Zhorov and Tikhonov, 2004). A residue is labeled by 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. B. The sequence alignment of segments involved in state-dependent contacts of residues in position i28 and i32. Underlined residues are mutations explored in this work.

Fig. 2. Voltage dependence of activation and inactivation of BgNa_v1-1a and mutant sodium channels. A. Voltage dependence of activation. B. Voltage dependence of inactivation. Voltage step protocols used to generate these curves are indicated above each panel.

Fig. 3.

Effects of D^{3i28V} and E^{3i32G} mutations on the sensitivity of BgNaV1-1a channels to permethrin (PMT) and deltamethrin (DMT). A and B. Tail currents induced by PMT or DMT from oocytes expressing BgNaV1-1a and mutant channels. C. Percentage of channel modification by PMT and DMT. The values of EC₂₀ for permethrin were 0.13, 0.63, 0.12 and 0.49 μM for BgNaV1-1a, D^{3i28V}, E^{3i32G} and D^{3i28V}/E^{3i32G} channels, respectively. The values of EC₂₀ for deltamethrin were 0.09, 0.59, 0.07 and 0.08 μM for BgNaV1-1a, D^{3i28V}, E^{3i32G} and D^{3i28V}/E^{3i32G} channels, respectively. The number of oocytes for each mutant construct was >5. Error bars indicate mean ± SEM. The asterisks indicate significant differences from the BgNaV1-1a channel as determined by using one-way analysis of variance (ANOVA) with Scheffé's *post hoc* analysis (*p* < 0.05).

Fig. 4. Effects of D^{3i28V} and E^{3i32G} mutations on the sensitivity of BgNa_v1-1a channels to cypermethrin (CPMT) and NRDC 157. A and B. Tail currents induced by NRDC 157 or CPMT from oocytes expressing BgNa_v1-1a and mutant channels. C. Percentage of channel modification by 1.0 μM NRDC 157 and 1.0 μM CPMT. The number of oocytes for each mutant construct was >5. Error bars indicate mean ± SEM. The asterisks indicate significant differences from the BgNa_v1-1a channel as determined by using one-way ANOVA with Scheffé's *post hoc* analysis (*p* < 0.05).

Fig. 5. Side views of the closed-state (A) and open-state (B) models of the wildtype BgNa_v1-1a channel and the open-state modes of the double-mutant D³ⁱ²⁸V/E³ⁱ³²G (lower panes at B). Repeats I, II, III and IV are colored pink, yellow, green and gray. In the open channel, E³ⁱ³² lacks inter-segment contacts, while D³ⁱ²⁸ forms unfavorable contact with the hydrophobic part of S^{3o5}. In the open channel, both D³ⁱ²⁸ and E³ⁱ³² are engaged in the inter-segment H-bonds with Q^{3o1} and N^{3k9}. In the double mutant these H-bonds are lost, which would increase mobility of the cytoplasmic part of IIIS6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Voltage dependence of activation and inactivation of BgNa_v1-1a and mutant sodium channels.

Na ⁺ channel type	Activation		Inactivation		n
	V _{1/2} (mV)	k (mV)	V _{1/2} (mV)	k (mV)	
BgNa _v 1-1a	-30.2 ± 0.8	3.8 ± 0.2	-50.9 ± 0.4	4.5 ± 0.1	15
D ³²⁸ V	-15.3 ± 0.9 *	6.8 ± 0.3	-48.3 ± 0.4	5.1 ± 0.1	22
E ³³² G	-27.3 ± 0.8	4.6 ± 0.2	-50.2 ± 0.3	5.2 ± 0.1	25
D ³²⁸ V/E ³³² G	-9.4 ± 0.8 *	8.1 ± 0.3	-50.5 ± 0.4	6.4 ± 0.2	25

V_{1/2} is the voltage for half-maximal conductance or inactivation, k is the slope factor for conductance or inactivation.

The value in the table represent the mean ± SEM, and n is the number of oocytes used.

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

Table 2

The tail currents decay of BgNa_v1-1a and mutant channels induced by deltamethrin (DMT) and cypermethrin (CPMT).

Na ⁺ channel type	Integral modified channels (%)		n
	Deltamethrin	Cypermethrin	
BgNa _v 1-1a	459 ± 25	465 ± 29	9
D ³ⁱ²⁸ V	47 ± 5 ^a	55 ± 5 ^a	10
E ³ⁱ³² G	438 ± 30	440 ± 35	8
D ³ⁱ²⁸ V/E ³ⁱ³² G	283 ± 31 [*]	182 ± 29 [*]	10

The value in the table represent the mean ± SEM, and n is the number of oocytes used.

^{*} significantly different from the wildtype channel using one-way ANOVA with Scheffé's post hoc analysis (p < 0.05).

^aPercentage of integral channel modification of D³ⁱ²⁸V mutant channel by DMT or CPMT, the tail currents induced by DMT or CPMT on D³ⁱ²⁸V channels were too small, it's unfeasible to compare the decay of pyrethroid-induced tail currents between wildtype and D³ⁱ²⁸V channels using this method.