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A residue in the transmembrane segment 6 of domain I in insect and mammalian sodium channels regulate differential sensitivities to pyrethroid insecticides

Eugênio E. Oliveira[†], Yuzhe Du, Yoshiko Nomura, and Ke Dong^{*}

Department of Entomology, Genetics and Neuroscience Programs; Michigan State University; East Lansing, MI 48824, USA

Abstract

Voltage-gated sodium channels are critical for electrical signaling in the nervous system. Pyrethroid insecticides exert their toxic action by modifying the gating of sodium channels. A valine to methionine mutation in the transmembrane segment 6 of domain I (IS6) of sodium channels from tobacco budworms (*Heliothis virescens*) has been shown to alter channel gating and reduce insect sodium channel sensitivity to pyrethroids. A valine to leucine substitution was subsequently reported in pyrethroid-resistant bedbug populations. Intriguingly, pyrethroid-resistant mammalian sodium channels possess an isoleucine at the corresponding position. To determine whether different substitutions at this position alter channel gating and confer pyrethroid resistance, we made valine to methionine, isoleucine or leucine substitutions at the corresponding position, V409, in a cockroach sodium channel and examined the gating properties and pyrethroid sensitivity of the three mutants in *Xenopus* oocytes. All three mutations reduced the channel sensitivity to three pyrethroids (permethrin, cismethrin and deltamethrin). V409M, but not V409I or V409L, caused 6–7 mV depolarizing shifts in the voltage dependences of both activation and inactivation. V409M and V409L slowed channel activation kinetics and accelerated open-state deactivation kinetics, but V409I did not. Furthermore, the substitution of isoleucine with valine, but not with methionine nor leucine, at the corresponding position in a rat skeletal muscle sodium channel, rNa_v1.4, enhanced channel sensitivity to deltamethrin. Collectively, our study highlights an important role of residues at 409 in regulating not only sodium channel gating, but also the differential sensitivities of insect and mammalian sodium channels to pyrethroids.

Keywords

voltage-gated ion channels; pyrethroids; knockdown resistance; kdr mutations

1. Introduction

Pyrethroids are a large class of structurally diverse, synthetic analogues of natural pyrethrins from the flower extracts of *Tanacetum cinerariaefolium* (Elliott, 1977). They are broad-spectrum insecticides and are used worldwide in the control of virtually all agriculturally

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^{*}Corresponding author: dongk@msu.edu, Phone: (517)-432-2034, Fax: (517)-353-4354.

[†]Present address: Department of Entomology; Federal University of Viçosa (UFV), Viçosa-MG, 36570-000, Brazil

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and medically important arthropod pests, including bedbugs and mosquitoes. Pyrethroids exert toxic effects by modifying the gating kinetics of sodium channels thereby disrupting the normal electrical signaling in nerve and other excitable cells (Bloomquist and Soderlund, 1988, Bloomquist, 1996, Narahashi, 1988, Narahashi, 2000).

A serious threat to the sustained use of pyrethroids and other insecticides is the development of resistance (Butler, 2011, Denholm et al., 2002, Hemingway et al., 2002, Hemingway and Ranson, 2000, Li et al., 2007, Nauen, 2007, Soderlund and Bloomquist, 1990). Understanding the molecular bases of insecticide action and resistance in arthropod pests are crucial for early detection and monitoring of resistance and development of appropriate strategies to delay the further spread of resistance. A major mechanism of pyrethroid resistance, known as knockdown resistance (kdr) (Soderlund and Bloomquist, 1990), is caused by mutations in sodium channels. Since its initial report in the house fly (Milani, 1954), kdr has been documented globally in almost all medically and agriculturally significant arthropod pests (Soderlund 2005; 2012, Rinkevich et al., 2013). So far, more than a dozen kdr mutations detected in diverse pyrethroid-resistant arthropod pests have been confirmed to confer pyrethroid resistance in the *Xenopus* oocyte expression system (Davies et al., 2007, Dong, 2007, Soderlund, 2012, Rinkevich et al., 2013). Information accumulated from the functional characterization of pyrethroid resistance mutations began to contribute to the general knowledge of the gating mechanisms and the molecular basis of the differential sensitivity between insect and mammalian sodium channels (Du et al., 2009b, Du et al., 2010, Du et al. 2012, O'Reilly et al., 2006, Usherwood et al., 2007, Vais et al. 1997).

Like their mammalian counterparts, the insect sodium channel is a large transmembrane protein having four homologous domains (I-IV), each domain possessing six transmembrane segments (S1-S6). S4s along with S1-S3 segments constitute the voltage-sensing modules, whereas S5, S6, and the membrane-reentrant loop (called the P-region) connecting S5 and S6 segments form the pore module. In response to membrane depolarization, S4 segments move outward, initiating the opening of sodium channels (i.e., the opening of the activation gate). This process is called activation. Based on the crystal structures of the closed potassium channels (Doyle et al., 1998, Jiang et al., 2002) and the bacterial sodium channel Na_vAb (Payandeh et al., 2011), the activation gate is located at the cytoplasmic end of the pore, where all four S6 segments converge. After a brief opening, the sodium channel is fast-inactivated, which is achieved by the movement of an inactivation particle (formed mainly by residues in the short intracellular linker connecting domains III and IV), which physically occludes the open pore. Upon repolarization, the sodium channel recovers from inactivation and deactivates (i.e., closing of the activation gate).

Multiple kdr mutations have been found in the transmembrane segments, mainly in IS6, IIS5, IIS6 and IIIS6. Additional kdr mutations have also been found in the linkers connecting the transmembrane segments or domains, including the linker connecting S4-S5 of domain II, and the linkers connecting domains I and II, and domains III and IV (Dong, 2007, Soderlund, 2005, Rinkevich et al., 2013). Computer modeling of the pyrethroid receptor site(s) in the house fly sodium channel using crystal structures of potassium channels showed that a pyrethroid receptor site is formed by residues in IIS4-S5, IIS5 and IIIS6 at the II/III interface (O'Reilly et al., 2006). According to this model, some kdr mutations are located close to or within the pyrethroid receptor site and possibly confer resistance by reducing pyrethroid binding. These kdr mutations include the M918T mutation (amino acid numbering based on the house fly sodium channel) in IIS4-S5, three mutations (T929I, L925I and L932F) in IIS5, and an F1538I mutation in IIIS6. However, many kdr mutations are not situated near the pyrethroid receptor site (O'Reilly et al., 2006). One of these kdr mutations is a valine to methionine change (V410M, based on the amino acid

numbering of the house fly sodium channel) in IS6 from pyrethroid-resistant tobacco budworms, *Heliothis virescens* and other substitutions including a valine to leucine mutation in pyrethroid-resistant bedbugs, *Cimex lectularius* (Hopkins and Pietrantonio, 2010, Yoon et al., 2008, Zhu et al., 2010). The mechanism by which these mutations confer pyrethroid resistance remains unknown. It has been previously suggested that sodium channel mutations could also confer pyrethroid resistance by altering channel gating or allosteric modulation thereby counteracting the action of pyrethroids (Davies et al., 2007, Du et al. 2010, Vais et al., 1997, Soderlund 2012).

Differential sensitivities of mammalian sodium channels to pyrethroids have been well documented (Narahashi 2000; Soderlund, 2012). Some mammalian sodium channel isoforms are virtually insensitive to pyrethroids, such as the rat brain rNa_v1.2 channel, the rat skeletal muscle rNa_v1.4 channel and the rat Na_v1.7 channel which is mainly expressed in the peripheral nervous system (Peng et al., 2009, Smith and Soderlund, 1998, Soderlund, 2012, Vais et al., 2001, Vais et al., 2000, Wang et al., 2001). While V410 in IS6 is conserved in all insect sodium channel sequences that are available in the GenBank, the residue corresponding to V410 is variable in mammalian sodium channels. A valine is present in Na_v1.3, Na_v1.5, Na_v1.6 and Na_v1.8 channels (Fig. 1B). However, an isoleucine is found in Na_v1.1, Na_v1.2, Na_v1.4 and Na_v1.7 channels, such as I433 in rNa_v1.4, and a leucine in Na_v1.9 (Fig. 1B). Because Na_v1.2, Na_v1.4 and Na_v1.7 are more resistant to pyrethroids than Na_v1.3, Na_v1.6 and Na_v1.8, it has been predicted that sequence variations observed at this position contribute to the different sensitivities of mammalian sodium channels to pyrethroids (Soderlund, 2012). However, no functional analyses have been performed in either insect or mammalian sodium channels to test this hypothesis.

In this study, we made valine to methionine, isoleucine or leucine substitutions, V409M, V409I and V409L, in a cockroach sodium channel, BgNa_v1-1a, at the position corresponding to V410 in the house fly sodium channel. We examined the effects of these substitutions on channel gating and channel sensitivity to pyrethroids. Our results show that variations in the side chain of the residue at this position have different effects on channel gating and channel sensitivity to pyrethroids, demonstrating the importance of V409 in both channel gating and pyrethroid action. Furthermore, our mutational analysis using rNa_v1.4 revealed that I433 (corresponding to V409 in BgNa_v1-1a) contributes to pyrethroid resistance in the rNa_v1.4 channel.

2. Materials and methods

2.1. Site-directed mutagenesis

The cockroach sodium channel, BgNa_v1-1a (pyrethroid-sensitive) was used to generate valine to leucine, isoleucine and methionine mutants (V409L, V409I and V409M, Fig. 1A). The rat skeletal sodium channel, rNa_v1.4, was used to generate isoleucine to valine, methionine and leucine mutants. The rNa_v1.4 plasmid was kindly provided by G.K. Wang (Harvard Medical School, Boston). Site-directed mutagenesis was performed by polymerase chain reaction (PCR) using mutant primers and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). The mutagenesis result was verified by DNA sequencing.

2.2. Expression of BgNa_v and rNa_v1.4 channels in *Xenopus laevis* oocytes

The procedures for oocyte preparation and cRNA injection were identical to those described previously (Du et al. 2009b, Tan et al., 2002). For robust expression of BgNa_v1-1a sodium channels, cRNA was co-injected into oocytes with cRNA encoding the *Drosophila melanogaster* tipE auxiliary subunit (1:1 ratio), which enhances the expression of insect sodium channels in oocytes (Feng et al., 1995, Warmke et al., 1997).

2.3. Characterization of channel sensitivity to pyrethroid insecticides

The effects of pyrethroids were quantified by measuring pyrethroid-induced tail currents using two-electrode voltage clamp. Electrophysiological recordings and data analysis were similar to those described previously (Tan et al. 2002, Tan et al., 2005). Briefly, the pyrethroid-induced tail current was recorded during a 100-pulse train of 5-ms step depolarizations from -120 to 0 mV with 5-ms interpulse intervals (Vais et al. 2000). The percentage of channels modified by pyrethroids was calculated using the equation $M = \{ [I_{\text{tail}} / (E_h - E_{\text{Na}})] / [I_{\text{Na}} / (E_t - E_{\text{Na}})] \} \times 100$ (Tatebayashi and Narahashi, 1994), 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 step depolarization.

Pyrethroid-induced tail currents were measured with a Warner OC725C oocyte clamp (Warner Instrument, Hamden, CT, USA) and processed with a Digidata 1322A interface (Axon Instruments Inc., Foster City, CA, USA). Data were sampled at 50 kHz and filtered at 2 kHz. Leak currents were corrected by p/4 subtraction (Armstrong and Bezanilla, 1974). pClamp 9.2 software (Axon Instruments Inc., Foster City, CA, USA) was used for data acquisition and analysis. A disposable perfusion system developed by Tatebayashi and Narahashi (1994) was used to apply pyrethroid insecticides in a home-made disposable recording chamber. Effects of pyrethroids were measured 10 min after the application of insecticide.

2.4. Cell-attached macropatch recording and analysis

Voltage dependences of activation and fast inactivation, channel activation and deactivation kinetics were recorded using the cell-attached macropatch technique. Methods for cell attached macropatch recordings and data analysis are similar to those described in Groome et al. (1999). Briefly, before macropatch recording, vitelline membranes were manually removed following a brief hyperosmotic treatment in a solution containing (in mM): 96 NaCl; 2 KCl; 20 MgCl₂; 5 Hepes; 400 mannitol. The pH was adjusted to 7.4. Electrodes with tip resistance between 0.5 and 1.2 MΩ were filled with a solution containing (in mM): 96 NaCl; 4 KCl; 1 MgCl₂; 1.8 CaCl₂; 5 HEPES. Bath solution used was (in mM): 9.6 NaCl; 88 KCl; 11 EGTA; 5 HEPES, pH 7.4. Both Pipette and bath solutions had their pH adjusted to 7.4. Voltage clamping and data acquisition were done using an Axopatch 200B amplifier (Molecular Devices, Foster City, CA, USA) and processed with a Digidata 1322A interface (Axon Instruments Inc., Foster City, CA, USA). Data were sampled at 50 kHz and filtered at 2 kHz. Leak currents were corrected by p/4 subtraction (Armstrong and Bezanilla, 1974). pClamp 9.2 software (Axon Instruments Inc., Foster City, CA, USA) was used for data acquisition and analysis.

The voltage dependence of sodium channel conductance (G) was calculated by measuring the peak current at test potentials (50 ms) ranging from -80 mV to $+65$ mV in 5-mV increments and divided by the driving force ($V - V_{\text{rev}}$), where V is the test potential and V_{rev} is the reversal potential of the current. Peak conductance values were normalized to the maximal peak conductance (G_{max}) and fitted with a two-state Boltzmann equation of the form $G/G_{\text{max}} = [1 + \exp((V - V_{1/2})/k)]^{-1}$, 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. Voltage dependence of fast inactivation was determined by using 100-ms inactivating prepulses ranging from -150 mV to 0 mV in 5-mV increments from a holding potential of -150 mV, followed by test pulses to -10 mV for 50 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 $I/I_{\text{max}} = [1 +$

$(\exp((V - V_{1/2})/k))^{-1}$ 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 voltage for half-maximal activation, and k is the slope factor.

Activation kinetics (time constant, τ_m) were measured by monoexponentially fitting the rising phase (10% to 90% of peak current) of the inward sodium current during 50-ms step commands to voltages ranging from -40 to $+20$ mV in 5-mV increments. To measure channel deactivation, channels were opened with a brief 0.25 ms depolarization to 0 mV, and tail currents were elicited by repolarizing commands to voltages ranging from -190 to -40 mV. Deactivation time constants (τ_D) were derived from the monoexponential decay of tail currents according to the function: $I(t) = \text{offset} + a_1 \exp(-t/\tau_D)$, where $I(t)$ is current amplitude as a function of time, *offset* is the plateau amplitude (asymptote), a_1 is current amplitude at time 0, and τ_D is the time constant. Oocytes with a peak sodium current greater than 8 μA from the two-electrode voltage clamp were used in the analysis of activation and deactivation kinetics.

2.5. Chemicals

Deltamethrin, permethrin and cismethrin were kindly provided by Dr. Bhupinder Khambay (Rothamsted Research Institute, Hertfordshire, UK), Dr. Ralf Nauen (Bayer CropScience AG, Monheim, Germany) and Dr. John Clark (University of Massachusetts, Amherst MA, USA), respectively. The purities of different pyrethroids range from 99.3% to 99.8%. Stock solutions of pyrethroids (100 mM) were dissolved in dimethyl sulfoxide (DMSO). The working concentration was prepared in ND96 recording solution just prior to the experiments. The concentration of DMSO in the final solution was $<0.5\%$, which had no effect on the function of sodium channels in the experiments.

Data are presented as mean $\pm$ S.D. Statistical analysis was performed using a one-way ANOVA test and Scheffe's post hoc analysis. Significance values were set at $P < 0.05$ or as indicated in the table and figure legends.

3. Results

3.1. All three mutations confer BgNa_v1-1a channel resistance to pyrethroids

Pyrethroid insecticides prolong the opening of sodium channels, which is evident in slowly-decaying tail currents associated with repolarization in voltage-clamp experiments (Narahashi, 1996, Vijverberg et al., 1982). Because pyrethroids preferably interact with the open state of insect sodium channels (Vais et al., 2000), to elicit pyrethroid-induced tail currents, we used a protocol of 100-pulse trains of 5-ms step depolarizations from -120 to 0 mV with 5-ms interpulse intervals (Fig. 2A). Decay of the tail current induced by deltamethrin (a type II pyrethroid) in BgNa_v1-1a channels was slow and biphasic (Table 1, Fig. 2A), whereas tail currents induced by permethrin and cismethrin (both type I pyrethroids) had a rapid and monophasic decay (Table 1, Fig. 2A). There is no difference between BgNa_v1-1a and V409M/L/I mutant channels in the kinetics of decay of the pyrethroid-induced tail currents (Table 1).

To assess the potency of insecticides on BgNa_v1-1a and mutant channels, we determined the percentage of channel modified by the insecticides (Fig. 2B). Consistent with results from earlier studies (Lee et al., 1999, Lee and Soderlund, 2001, Liu et al., 2002, Zhao et al., 2000), the V409M mutation reduced the sensitivity of the BgNa_v1-1a channel to all three pyrethroids (Fig. 2B). Similarly, the V409L mutation drastically reduced BgNa_v1-1a channel sensitivity to all three pyrethroids. Although the V409I mutation also reduced BgNa_v1-1a sensitivity to all three pyrethroids, the reduction in pyrethroid sensitivity by this mutation is less drastic than other two mutations (Fig. 2B).

We also used another protocol without conditioning pulses (Fig. 2C) to assess the effects of the three mutations on resting channel modification by pyrethroids. Consistent with the results from previous studies (Smith and Soderlund, 1998, 2001, Soderlund and Lee, 2001), both cismethrin and permethrin, but not deltamethrin, caused significant resting channel modification (Fig 2C). Furthermore, all three mutations reduced resting channel modification.

3.2. V409M, but not V409L and V409I, mutation altered the voltage dependences of activation and fast inactivation

We used the cell-attached macropatch technique to determine the effects of the V409L, V409I, or V409M mutations on the gating properties of the BgNa_v1-1a channel. The maximal value of the peak sodium current in the patches varied ranging from 50 pA to 1.2 nA. We assessed the voltage dependence of activation of BgNa_v1-1a, V409L, V409I and V409M channels by measuring the amplitude of the peak sodium channels obtained following a 50-ms depolarization from the holding potential of -150 mV to test potentials ranging from -80 mV to +65 mV (Fig. 3A). The V409M mutation caused a 6-mV depolarizing shift in the voltage dependence of activation of BgNa_v1-1a channels (Fig. 3B, Table 2 $P < 0.05$), whereas V409L and V409I mutations did not significantly alter the voltage dependence of activation of BgNa_v1-1a channels (Fig. 3B, Table 2, $P > 0.05$).

We also assessed the effects of the mutations on the voltage dependence of fast inactivation (Fig. 3C). The V409M mutation shifted the voltage dependence of inactivation of BgNa_v1-1 channels by 7 mV in the depolarizing direction (Fig. 3C, Table 2, $p < 0.05$). In contrast, V409L and V409I mutations did not significantly affect the voltage dependence of fast inactivation (Fig. 3C, Table 2, $P > 0.05$).

3.3. V409L and V409M, but not V409I, mutations slowed activation and accelerated deactivation kinetics

To examine the effect of V409L and V409M mutations on the kinetics of activation, we determined the activation time constant (τ_m) values by fitting the rising phase (10% to 90%) of the inward sodium currents with a monoexponential fit and plotted as a function of the test potential (Fig. 4). Both the V409L and V409M mutations slowed the activation rate over almost the entire voltage range tested (-40 mV to +15 mV, $p < 0.05$) (Fig. 4B). In contrast, the V409I mutation did not (Fig. 4B).

The molecular determinants involved in voltage-dependent activation are likely also involved in regulating channel deactivation (Groome et al. 1999, Kontis et al. 1997). We used the protocol shown in Fig. 5A to measure the time course of channel deactivation. Channels were activated by a brief depolarization to 0 mV for 0.25 ms (before a significant amount of fast-inactivation could occur) followed by hyperpolarization to a membrane potential between -190 mV and -40 mV for 20 ms. We chose the voltage range from -190 mV to -60 mV because at this voltage range the tail current is largely due to deactivation, not inactivation (Featherstone et al. 1998). Therefore, the tail current associated with the repolarization after the brief depolarization largely reflects open-state deactivation (i.e., closing of the activation gate). A single exponential fit of the decaying tail current was used to measure deactivation time constants (τ_D), and deactivation time constants were plotted as a function of the test potential (Fig. 5B).

The V409L mutation significantly accelerated open state deactivation over the command voltage range between -190 mV and -90 mV. The V409M mutant channels exhibited a similar acceleration of channel deactivation, with a command voltage range from -190 mV

and -80 mV (Fig. 5B). In contrast, the V409I mutation did not significantly alter the deactivation kinetics compared with the wild type channel (Fig. 5B).

3.5. Valine substitution of isoleucine in rNa_v1.4 enhanced the channel sensitivity to deltamethrin

Reduced sensitivity of the V409I channel to pyrethroids suggests possible involvement of the corresponding isoleucine in pyrethroid-insensitive mammalian sodium channels in pyrethroid resistance. Therefore, we directly test this prediction in the rNa_v1.4 channel. We replaced the corresponding I433 in the rNa_v1.4 channel with valine, leucine or methionine and examined the sensitivity of the mutant rNa_v1.4 channels to deltamethrin. As shown in Fig. 6, the I433V mutation significantly increased the sensitivity of the rNa_v1.4 channel to deltamethrin, whereas the I433M/L mutations did not.

4. Discussion

In this study we found that three different substitutions at V409 in IS6 of a cockroach sodium channel all reduced channel sensitivity to two type I and one type II pyrethroids. Our results also showed that V409 is important for channel gating. However, the three substitutions have different effects on the voltage dependence of activation, activation kinetics and deactivation kinetics, suggesting that altered channel gating per se may not be exclusively associated with pyrethroid resistance. Furthermore, we found that the valine to isoleucine substitution at the corresponding position in the rNav1.4 sodium channel confers pyrethroid resistance. Taken together, our results show an important role of V409 in regulating not only sodium channel gating, but also pyrethroid sensitivity in both insect and mammalian sodium channels.

Several *kdr* mutations have previously been shown to shift the voltage-dependence of activation in the depolarizing direction. For example, the leucine to phenylalanine or histidine mutation in IIS6 at position 1014 (based on the amino acid numbering of the house fly sodium channel) shifted the voltage dependence of activation in the depolarizing direction (Burton et al., 2011). Similarly, the voltage-dependence of sodium channel activation in neurons from pyrethroid resistant *H. virescens* adults carrying the V409M mutation is also shifted by about 13 mV in positive depolarizing direction (Lee et al., 1999), consistent with our findings in this study. A depolarizing shift in the voltage-dependence of activation indicates that these *kdr* mutant channels need a stronger depolarization to open. Because pyrethroids preferentially bind to sodium channels in the open conformation (Du et al., 2009a, O'Reilly et al. 2006, Usherwood et al. 2007), the V409M and L1014F/H channels are expected to have a reduced channel open probability. If so, the V409M and L1014F/H mutations could confer pyrethroid resistance by reducing the chance of pyrethroid action on sodium channels. However, we found that, in contrast to the V409M mutation, the V409L/I mutations did not alter the voltage-dependence of activation (Table 2), yet the V409L mutation confers a similar level of pyrethroid resistance in *Xenopus* oocytes (Fig. 2B). These results suggest that a positive shift in the voltage dependence of activation *per se* may not be the principle cause of pyrethroid resistance in these mutant sodium channels.

Pyrethroids prolong the opening of sodium channels by enhancing channel activation and inhibiting channel deactivation and inactivation (Narahashi, 1996, Vijverberg et al. 1982). Theoretically, slowed channel activation (opening) and accelerated channel deactivation (closing) by *kdr* mutations could counteract the action of pyrethroids resulting in pyrethroid resistance. A potential link between altered gating kinetics and pyrethroid resistance has been previously reported (Du et al. 2010). A substitution of an aspartic acid (D) with lysine (K) at the position 802 in IIS1 in a BgNa_v splicing variant caused slowed activation and accelerated deactivation kinetics (Du et al. 2010). Interestingly, the D802K substitution also

reduced channel sensitivity to pyrethroids, but did not affect pyrethroid binding (Du et al. 2010). Although V409M/L mutations slowed activation and accelerated deactivation, a strong association between altered gating and pyrethroid resistance cannot be established because the V409I mutation did not alter these gating properties, but also confers pyrethroid resistance. However, since the V409I mutation provides less protection to pyrethroids than the V409M/L mutations (Fig. 2B), the altered gating by the V409M/L mutations could contribute to the higher levels of pyrethroids resistance. Overall, findings in this study suggest that there is likely a channel gating-independent mechanism for pyrethroid resistance caused by mutations at V409. It is possible that these mutations at V409 interfering with the binding of pyrethroids to their receptor site(s), either directly or via an allosteric mechanism.

V409 is located in the middle of IS6 and is the fifth residue downstream of the putative hinge residue that permits the hinged motion of the S6 segments during pore opening (Jiang et al. 2002). The movement of S6 segments in response to voltage changes dictates channel opening/closing and is coupled to the movement of the S4 voltage-sensors (Jiang et al. 2002; Payandeh et al., 2011). It is not surprising, therefore, that mutations in S6s have a profound influence on the voltage dependence of activation, channel activation and deactivation kinetics. Amino acid substitutions within IS6 in mammalian sodium channels have been documented to affect channel gating resulting in altered voltage dependence, altered kinetics, and disease (Fischer et al., 2009, Kondratiev and Tomaselli, 2003, Nau et al., 1999, Wang and Wang, 1997). For example, I425C and I436C mutations in the rat sodium channel rNa_v1.4, corresponding to I401 and I412 in BgNa_v1-1a, result in depolarizing shifts in the voltage dependence of activation (Kondratiev and Tomaselli, 2003). Similarly, substitution of asparagine with alanine at position 434 (N434A) in rNa_v1.4, corresponding to N410 in BgNa_v1-1a, causes a depolarizing shift in the voltage dependence of activation (Nau et al. 1999, Wang and Wang, 1997), whereas the N434C substitution shifted the voltage dependence of activation in the hyperpolarizing direction (Kondratiev and Tomaselli, 2003). A valine to methionine near the C terminus of IS6 (V400M) of human sodium channel hNa_v1.7, corresponding to V416 in BgNa_v1-1a, shifts the voltage dependence of activation in the hyperpolarizing direction and slows the deactivation kinetics, which leads to the pain syndrome called inherited erythromelalgia (Fischer et al. 2009). Given the importance of IS6 in proper channel gating, we speculate that V409L/M mutations alter the position or the movement of IS6 in response to membrane depolarization, thereby impeding channel opening and accelerating channel closing, resulting in slowed channel activation and accelerated deactivation kinetics.

Pyrethroid insecticides have been used widely to control arthropod pests and human disease vectors because of their high insecticidal activity and relatively low mammalian toxicity. The differential sensitivity between insect and mammalian sodium channels contributes to the selective toxicity of pyrethroid insecticides (Narahashi 2000; Soderlund 2012). Differential sensitivity of mammalian sodium channels to pyrethroids was first documented in the dorsal root ganglia by Narahashi and associates (Ginsburg and Narahashi, 1993, Tabarean and Narahashi, 1998, Tatebayashi and Narahashi, 1994). Subsequent functional characterization of mammalian sodium channels *in vitro* expression systems revealed that some of mammalian sodium channel isoforms are virtually insensitive to pyrethroids (Soderlund, 2012). So far, the lower sensitivity of mammalian sodium channel isoforms compared with insect sodium channels has been partially attributed to an amino acid polymorphism in the linker connecting S4-S5 of domain II. While pyrethroid-sensitive insect sodium channels have a methionine at this position (M918 in the house fly sodium channel), all mammalian channels have an isoleucine at the corresponding position (Soderlund et al., 2012). Substitutions of the isoleucine with methionine in rat Na_v1.2 (Peng et al. 2009, Vais et al. 2000a), rNa_v1.4 (Wang et al. 2001) or rNa_v1.8 (Soderlund and Lee

2001) channels significantly increased the sensitivity of these channels to pyrethroids. Here we showed an isoleucine in IS6 contributes to pyrethroid resistance in the rNa_v1.4 channel and likely in the other two pyrethroid-insensitive channels as well (Na_v1.2 and Na_v1.7) because they also have an isoleucine at this position (Fig. 1B). Furthermore, valine in Na_v1.3, Na_v1.5 and Na_v1.6 and Na_v1.8 channels likely contributes to the sensitivity of these channels to pyrethroids. Also it is worth mentioning that the Na_v1.9 channel has a leucine (Fig. 1B). Although the sensitivity of the Na_v1.9 channel to pyrethroids has not been examined, based on our results, we predict that this leucine likely also modulates the sensitivity of Na_v1.9 channels to pyrethroids.

5. Conclusion

Using site-directed mutagenesis coupled with functional analysis in *Xenopus* oocytes, we have confirmed that residues at 409 in the cockroach sodium channel and at the corresponding position in a rat skeletal muscle sodium channel regulate different sensitivities of insect and mammalian sodium channels to pyrethroids. Once again naturally-occurring pyrethroid resistance mutations in insect sodium channels have proven to be valuable resources in the elucidation of the molecular basis of selective toxicity of pyrethroids on insect and mammalian sodium channels. Further studies aimed at understanding how these mutations modulate pyrethroid sensitivity would further advance the understanding of pyrethroid action on voltage-gated sodium channels at the molecular level.

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Highlights

V409M/L/I mutations in a cockroach sodium channel reduced the cockroach channel sensitivity to pyrethroids.

The V409M mutation altered the voltage dependence of activation and inactivation.

V409M and V409L altered the kinetics of activation and deactivation.

I433V (corresponding to V409) in a skeletal muscle sodium channel enhanced the channel sensitivity to deltamethrin, a pyrethroid insecticide.

Fig.1.

Pyrethroid resistance-associated amino acid substitutions in insect and mammalian sodium channels in IS6. (A) Schematic representation of the cockroach sodium channel protein (BgNav_v1-1a) indicating two knockdown resistance mutations in IS6. Sodium channels are large transmembrane proteins with four homologous repeats (I-IV), each having six transmembrane segments (S1-S6). Residue positions correspond to the cockroach BgNav_v protein (GenBank number: U73583). (B) Amino acid sequence alignments in IS6 from the wild-type BgNav_v1-1a, and nine mammalian (rat) sodium channels. The residues in the mammalian sodium channels corresponding to V409 in the BgNav_v1-1a channel are boxed. Na_v1.2 (Smith and Soderlund, 1998, Vais et al., 2000b), Na_v1.4 (Wang et al. 2000), Na_v1.7 (Tan and Soderlund, 2011) channels are more resistant to pyrethroids than Na_v1.3 (Tan and Soderlund, 2009), Na_v1.6 (Tan and Soderlund, 2010) and Na_v1.8 (Choi and Soderlund, 2006, Soderlund and Lee, 2001) channels. The pyrethroid sensitivity of Na_v1.9 has not been undermined.

Fig. 2.

Effects of V409M, V409L and V409I mutations on the sensitivity of BgNa_v1-1a channels to pyrethroids. (A) Representative traces of tail currents induced by deltamethrin (1 μ M), permethrin (1 μ M) and cismethrin (1 μ M) from the BgNa_v1-1a channel expressed in oocytes. Tail currents were recorded in response to a 100-pulse train of 5 ms depolarization from -120 to -10 mV in a frequency of 67 Hz. (B and C) Use-dependent modification (B) and resting modification (C) of BgNa_v1-1a and mutant channels by deltamethrin, permethrin and cismethrin. The method for determining the percentage of channel modification is described in Materials and Methods. Asterisks indicate significant differences in relation to the BgNa_v1-1a channel (one-way ANOVA with Scheffe's post hoc analysis, $P < 0.05$).

Fig. 3.

Effects of V409L, V409M and V409I mutations on the voltage dependences of activation and fast inactivation of BgNa_v1-1a channels. (A) Representative current traces evoked in BgNa_v1-1a channels by 50-ms depolarizations from a holding potential of -150 mV to voltages varying from -80 mV to +65 mV in 5-mV increments. (B) The voltage dependence of activation was measured using the voltage protocol detailed in inset. Peak conductance was normalized to the maximum conductance, plotted versus voltage, and fitted with a Boltzmann equation to calculate the voltage of half activation. (C) For inactivation, peak transient currents were measured using 100-ms inactivating prepulses ranging from -150 mV to 0 mV in 5-mV increments from a holding potential of -150 mV, followed by test pulses to -10 mV for 50 ms. The data were obtained from the cell-attached macropatch recording.

Fig. 4.

Effects of V409L, V409M and V409I mutations on activation kinetics. (A) Representative current traces evoked in BgNa_v1-1a and V409M/L/I mutant channels. (B) Mean time constant (τ_m) values from single exponential fits to channel activation were plotted as a function of the voltage at which the current was recorded. The recoding protocol used was the same as that in Fig. 3B. The data were obtained from the cell-attached macropatch recording.

Fig. 5.

Effects of V409L, V409M and V409I mutations on deactivation kinetics. (A) The recording protocol and representative current traces evoked in BgNa ν 1-1a and V409M/L/I mutant channels. From a holding potential of -150 mV, oocytes were depolarized to 0 mV to activate sodium channels. After 0.25 ms, the oocytes were then repolarized to potentials ranging from -190 mV to -40 mV for 20 ms, eliciting tail currents. (B) Deactivation decay time was obtained from a single exponential fit to tail currents evoked by the protocol shown in Fig. 5A. Means of deactivation decay time ($\bar{x}$) from single exponential fits to channel deactivation were plotted as a function of the voltage at which the current was recorded. The data were obtained from the cell-attached macropatch recording.

Fig. 6.

Effects of I433V, I433M and I433L mutations on the sensitivity of rNa_v1.4 channels to deltamethrin. (A) Representative current traces evoked in rNa_v1.4 and I433V channels. (B) Modification of rNa_v1.4 and I433V/M/L mutant channels by deltamethrin. The I433V substitution, but not the I433M/L, enhances the sensitivity of rNa_v1.4 channel to deltamethrin. The method for determining the percentage of channel modification is described in Materials and Methods. Asterisks indicate significant differences in relation to the rNa_v1.4 channel (one-way ANOVA with Scheffe's post hoc analysis, $P < 0.05$).

Table 1
The rate of decay of pyrethroid-induced tail currents

	BgNa _v 1-1a	V409M	V409L	V409I
<i>deltamethrin</i>				
τ_1	1.866 ± 0.341	2.028 ± 0.405	2.373 ± 0.191	1.694 ± 0.596
τ_2	0.262 ± 0.098	0.196 ± 0.032	0.359 ± 0.188	0.243 ± 0.140
<i>permethrin</i>				
	0.387 ± 0.178	0.258 ± 0.030	0.162 ± 0.065	0.437 ± 0.176
<i>cismethrin</i>				
	0.296 ± 0.143	0.145 ± 0.029	0.278 ± 0.149	0.272 ± 0.020

τ_1 : the rate of decay for permethrin/cismethrin-induced tail currents using a monoexponential equation. τ_1 and τ_2 : the rate of decay for deltamethrin-induced tail currents using a double exponential equation. No significant differences were found between BgNa_v1-1a and mutant channels for pyrethroid-induced tail currents using one-way ANOVA.

Table 2

Voltage dependence of activation and fast inactivation of BgNav_v1-1a and mutant channels.

Channel type	n	activation		inactivation	
		V _{1/2}	k	V _{1/2}	k
<i>BgNav_v1-1a</i>	19	-23.2 ± 4.8	8.5 ± 1.4	-63.9 ± 3.1	4.9 ± 0.3
<i>V409I</i>	12	-28.5 ± 5.1	7.3 ± 1.6	-64.4 ± 3.6	4.8 ± 0.6
<i>V409L</i>	7	-26.0 ± 2.9	8.0 ± 1.0	-65.9 ± 5.2	4.6 ± 0.6
<i>V409M</i>	14	-16.9 ± 5.2 [*]	8.0 ± 1.1	-56.7 ± 5.1 [*]	4.7 ± 0.5

^{*} denotes a statistically significant difference from BgNav_v1-1a using one-way ANOVA with Scheffé's post hoc analysis ($P < 0.05$)