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An alanine in segment 3 of domain III (IIIS3) of the cockroach sodium channel contributes to the low pyrethroid sensitivity of an alternative splice variant

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Abstract

In a previous study, we showed that two alternative exons (G1 and G2 encoding IIIS3-S4) were involved in the differential sensitivity of two cockroach sodium channel splice variants, BgNa_v1-1 and BgNa_v2-1 (previously called KD1 and KD2), to deltamethrin, a pyrethroid insecticide (Tan, et al., 2002b. Alternative splicing of an insect sodium channel gene generates pharmacologically distinct sodium channels. *J. Neurosci.* 22, 5300–5309.). Here, we report the identification of an amino acid residue in exon G2 that contributes to the low deltamethrin sensitivity of BgNa_v2-1. Replacement of A1356 in BgNa_v2-1 with the corresponding V1356 in BgNa_v1-1 enhanced the sensitivity of the BgNa_v2-1 channel to deltamethrin by six-fold. Conversely, substitution of V1356 with A1356 in BgNa_v1-1 produced a recombinant BgNa_v1-1 channel that was 5-fold more resistant to deltamethrin. These results demonstrate that A1356 contributes to the low sensitivity of BgNa_v2-1 to deltamethrin. A1356V substitution also shifted the voltage-dependence of activation by 10 mV in the hyperpolarizing direction. Possible mechanisms by which this amino acid change affects the action of pyrethroids on the sodium channel are discussed.

Keywords

Sodium channel; Pyrethroids; Alternative splicing

1. Introduction

Voltage-gated sodium channels are essential for the generation of the action potential in almost all excitable cells (Catterall, 2000). Sodium channels open in response to membrane depolarization (i.e., opening of activation gate), Na ions flux into the cell down the electrochemical gradient, further depolarizing the membrane, which is responsible for the rising phase of the action potential. A few milliseconds after channel opening, sodium channels inactivate and sodium ion influx stops, which partly contributes to the falling phase of the action potential. Subsequent recovery from inactivation and closure of the activation gate (i.e., deactivation) brings the sodium channel back to the resting state and ready to contribute to the initiation of another action potential.

Like mammalian counterparts, sodium channel proteins in insects contain four homologous domains (I–IV), each having six transmembrane segments (S1–S6) (Loughney et al., 1989). The structural features that are critical for mammalian sodium channel function are conserved in insect sodium channels. The S4 segments are believed to function as voltage sensors, which move outward in response to membrane depolarization, initiating the opening of sodium channels (Catterall, 2000). The S5–S6 segments are the pore-lining regions, defining the outer pore and the selectivity filter of the sodium channel (Catterall, 2000). The four domains are connected by three intracellular linkers. The first and second inter-domain linkers are more variable in sequence among different sodium channel proteins, whereas the third short linker connecting domains III and IV is more conserved and is critical for fast inactivation (Catterall, 2000).

Pyrethroids are a large class of synthetic insecticides structurally derived from naturally occurring pyrethrum extracted from *Chrysanthemum* species. These compounds exert their toxic effects by disrupting the function of voltage-gated sodium channels (Narahashi, 2000). Specifically, pyrethroids inhibit channel inactivation and deactivation, which causes prolonged opening of sodium channels and repetitive firing and nerve transmission block (Narahashi, 2000). In the past decade, several point mutations in insect sodium channels, naturally selected as a result of intensive use of pyrethroids, were found to reduce sodium channel sensitivity to pyrethroids and are responsible for knockdown resistance (*kdr*) in various insect species (Soderlund and Knipple, 2003). Identification of these *kdr* sodium channel mutations has significantly improved our understanding of the action of pyrethroids on sodium channels at the molecular level. The *kdr* mutations could reduce sodium channel sensitivity to pyrethroids by altering pyrethroid binding to the sodium channel or by changing channel gating properties to counteract the effects of pyrethroids. In support of the first mechanism, we recently showed that two *kdr* mutations, L993F and F1519I, in German cockroaches reduce pyrethroid binding to the pyrethroid receptor site (Tan et al., 2005).

Besides *kdr* mutations, we discovered that different cockroach sodium channel splice variants also exhibited different sensitivities to pyrethroids. For example, variant BgNa_v2-1 (previously called KD₂) with a mutually exclusive exon, G2, is 100-fold more resistant to deltamethrin (a type II pyrethroid) than variant BgNa_v1-1 (previously called KD₁) with a mutually exclusive exon, G1 (Tan et al., 2002b). Exon G1/G2 swapping experiment showed that exon G2 is partially responsible for the low sensitivity of BgNa_v2-1 to deltamethrin. There are 14 amino acid differences in IIS3-4 encoded by exon G1 and G2. To determine which amino acid residue(s) in exon G2 in BgNa_v2-1 is responsible for the low channel sensitivity to deltamethrin, we made a series of recombinant constructs by site-directed mutagenesis and examined the deltamethrin sensitivity of these recombinant channels expressed in *Xenopus* oocytes. Here, we report the identification of an amino acid residue that modulates variant-specific sodium channel sensitivity to deltamethrin.

2. Materials and methods

2.1. Site-directed mutagenesis

To make double and triple mutations of the residues that position next to each other (Fig. 1), a 1.4-kb *Eco*47III fragment which contains the G1 or G2 exon from BgNa_v1-1 or BgNa_v2-1, respectively, was subcloned into pAlter-1 vector and subjected to site-directed mutagenesis using the Altered Sites II in vitro Mutagenesis System (Promega, Madison, WI). We excluded L1346V and A1356V from the initial double and triple mutation analyses because of the conserved nature of the substitutions. After we found that none of the residues in the double and triple mutations were responsible for the differential sensitivity to deltamethrin, we made three single-mutation channels, BgNa_v1-1^{V1356A}, BgNa_v2-1^{A1356V}, and BgNa_v2-1^{L1346V}, and later a double mutant channel, BgNa_v2-1^{L1346V+A1356V}, by

polymerase chain reaction (PCR) using mutant primers and Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). All mutagenesis results were verified by DNA sequencing.

2.2. Expression of BgNa_v sodium channels in *Xenopus* oocytes

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

2.3. Two-electrode voltage-clamp recording and toxin application

All oocyte recordings were performed at room temperature (20–22 °C) in ND96 bath solution (96.0 mM NaCl; 2.0 mM KCl; 1.8 mM CaCl₂; 1.0 mM MgCl₂; 10.0 mM HEPES, adjust pH to 7.5 with 2 N NaOH). Recording electrodes were prepared from borosilicate glass using p-87 puller (Sutter instrument, Novato). Microelectrodes were filled with filtered 3 M KCl/0.5% agarose and had resistances between 0.4 and 1.0 MΩ. Currents were recorded and analyzed using the oocyte clamp instrument OC725C (Warner Instrument Corp., CT), Digidata 1322A interface (Axon Instrument, CA), and pClamp 8.2 software (Axon instruments, CA). The data were filtered at 2 kHz on-line and digitized at a sampling frequency of 20 kHz. Capacitance transients and leak currents were corrected by P/4 subtraction. The maximal peak sodium current was limited to <2.0 μA to achieve better voltage control by adjusting the amount of cRNA and the incubation time after injection. The voltage-dependence of activation and fast inactivation were determined using the protocols described in Tan et al. (2002a, b). The data were fitted with two-state Boltzmann equation to generate $V_{1/2}$, the midpoint of the activation or inactivation, and k , the slope factor.

Pyrethroids inhibit deactivation of sodium channels, consequently inducing large tail currents upon repolarization. The amplitude and decay of tail currents are two major parameters to quantify the action of pyrethroids. To record deltamethrin-induced tail currents, we applied a 100-pulse train of 5-ms depolarizations from –120 to –10 mV, as described by Vais et al. (2000). Deltamethrin preferably binds to sodium channels in the open state, so a 100-pulse train of 5-ms inter-pulse intervals was used to increase the availability of open channels. The method for deltamethrin application was identical to that described by Tan et al. (2002a, b). 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 sodium channels in the experiments. The deltamethrin induced tail currents were measured 10 min after toxin application using the protocol identical to that in Tan et al. (2002a, b). Percentages of channels modified by pyrethroid were calculated using the equation $M = \{I_{\text{tail}}/(E_h - E_{\text{Na}})/[I_{\text{Na}}/(E_t - E_{\text{Na}})]\} 100$ (Tatebayashi and Narahashi, 1994), where I_{tail} is the maximal tail current amplitude, E_h , E_{Na} and E_t are the holding potential, reversal potential and test potential, respectively. I_{Na} is the amplitude of the peak current during depolarization before pyrethroid exposure. Dose–response curves were fitted to the Hill equation: $M = M_{\text{max}}/[1 + (\text{EC}_{50}/[\text{pyrethroid}])^n]$, in which [pyrethroid] represents the concentration of pyrethroid and EC_{50} represents the concentration of pyrethroid that produced the half-maximal effect, n represents the Hill coefficient, and M_{max} is the maximal percentage of sodium channel modified. Because voltage-clamp fails at higher pyrethroid concentrations due to large leakage currents, we cannot obtain the upper portion of the dose–response curve. EC_{20} s were used to compare channel sensitivities among wild-type and mutant channels. The decays of tail currents were fitted with single- or double-exponential functions to determine time constants (Tau values),

with which tail current peak amplitudes were extrapolated at the time zero where there was no overlapping between capacitance and tail currents.

3. Results and discussion

BgNa_v1-1 and BgNa_v2-1 differ in the IIIS3-4 region encoded by two mutually exclusive exons G1 and G2, respectively, in addition to ten amino acid residue differences scattered throughout the sodium channel protein (Tan et al., 2002b). Exon swapping between BgNa_v1-1 and BgNa_v2-1 in an earlier study showed that exons G1/G2 are partially responsible for the differential sensitivity between BgNa_v1-1 and BgNa_v2-1 to deltamethrin (Tan et al., 2002b). There are 14 amino acid differences between exons G1 and G2 (Fig. 1A). To uncover the residue(s) in exons G1/G2 that are responsible for the different deltamethrin sensitivities, we made single, double, or triple amino acid substitutions (see Materials and methods for the details) in BgNa_v1-1 or BgNa_v2-1. Two single (L1346V and A1356V) and three double (LA → FV, IW → SL and RS → KT) substitutions were made in BgNa_v2-1. Two triple substitutions (GGQ → ADP and MMQ → VWE) were constructed in BgNa_v1-1. The resulting recombinant channels were expressed in *Xenopus* oocytes, the channel gating properties and channel sensitivity to two pyrethroids, permethrin (a type I pyrethroid) and deltamethrin (a type II pyrethroid), were examined.

We chose 1 μM deltamethrin for BgNa_v1-1 and its recombinant channels, and 10 μM for BgNa_v2-1 and its recombinant channels for the analysis of channel sensitivity to deltamethrin because BgNa_v2-1 is 100-fold more resistant to deltamethrin than BgNa_v1-1 (Tan et al., 2002b). As shown in Fig. 1B and C, the deltamethrin sensitivities of BgNa_v1-1^{ADP}, BgNa_v1-1^{VWE}, BgNa_v2-1^{FV}, BgNa_v2-1^{SL} and BgNa_v2-1^{KT} channels were similar to that of the parental channel BgNa_v1-1 or BgNa_v2-1. The sensitivity of the BgNa_v2-1^{A1356V} channel to deltamethrin, however, was enhanced significantly compared with BgNa_v2-1. BgNa_v2-1^{L1346V} channel was also more sensitive to deltamethrin than BgNa_v2-1, but the difference was subtle (Fig. 1C). These results demonstrate that A1356 is a primary contributor to the exon G2-specific low sensitivity to deltamethrin. We also replaced V1356 in the BgNa_v1-1 channel with an A. Consistent with the results from BgNa_v2-1^{A1356V}, we found that the V1356A mutation made the recombinant channel, the BgNa_v1-1^{V1356A}, more resistant to deltamethrin than the parental BgNa_v1-1 channel (Fig. 1B).

We next determined the level of change in channel sensitivity to deltamethrin by V1356A (in BgNa_v1-1) and A1356V (in BgNa_v2-1). A dose–response relationship was generated by plotting the percentage of channel modification against the deltamethrin concentration. The deltamethrin-induced tail currents recorded from oocytes expressing BgNa_v1-1^{V1356A}, BgNa_v2-1^{A1356V} and the parental channel are presented in Fig. 2. The time constants of the decay of tail currents of the recombinant channels were not significantly different from those of the parental channels (Table 2). However, the EC₂₀ values are 1.9 and 12.8 μM, respectively, for BgNa_v2-1^{A1356V} and BgNa_v2-1 channels. Thus, the BgNa_v2-1^{A1356V} channel was six-fold more sensitive to deltamethrin than the parental BgNa_v2-1 channel. The EC₂₀ values of BgNa_v1-1^{V1356A} and BgNa_v1-1 channels were 0.21 and 0.04 μM, respectively, representing a five-fold reduction in deltamethrin sensitivity of the BgNa_v1-1^{V1356A} channel, compared with that of the parental BgNa_v1-1 channel.

In the study by Tan et al. (2002b), we showed that swapping exons G1/G2 between BgNa_v1-1 and BgNa_v2-1 resulted in a 10-fold change in channel sensitivity to deltamethrin. Because the A1356V change in exon G2 alone did not produce a full 10-fold increase in deltamethrin sensitivity, we made a L1346V and A1356V double mutation in BgNa_v2-1 to determine whether these two changes have additive effects. However, we found that the

deltamethrin sensitivity of the BgNa_v2-1^{A1356V+L1346V} channel was similar to the BgNa_v2-1^{A1356V} channel. We speculate that other amino acid difference(s) between exons G1 and G2 are involved in enhancing the deltamethrin sensitivity of BgNa_v2-1^{A1356V}, even though they do not seem to alter channel sensitivity to deltamethrin by themselves (Fig. 1B). Further studies are needed to explore this possibility.

Based on their distinct poisoning symptoms, effects on sodium channels and the presence or absence of an α -cyano group in the 3-phenoxybenzyl alcohol moiety, pyrethroids are grouped into type I and type II. Type II pyrethroids, such as deltamethrin, possess this α -cyano group, whereas type I pyrethroids lack this group (Elliott, 1977). It is well established that type II pyrethroids modify sodium channel gating to a greater extent than type I pyrethroids (Narahashi, 1996, 2000). Both type I and type II pyrethroids prolong the open time of individual sodium channels, resulting in the induction of large tail currents associated with repolarization in whole cell recordings. However, type II pyrethroids induce channel opening for a much longer time than type I pyrethroids, leading to an extremely prolonged sodium current and membrane depolarization, which is followed by block of action potentials (Narahashi, 1996). The molecular basis underlying this difference remains to be determined. Consistent with the earlier findings (Tan et al., 2005), the permethrin (a type I pyrethroid)-induced tail currents decayed rapidly and were completely returned to the baseline at the end of recording, whereas the deltamethrin-induced tail current decayed very slowly (Fig. 3). We found that BgNa_v2-1 was also more resistant to permethrin than BgNa_v1-1 and that A1356 contributed to the reduced permethrin sensitivity of BgNa_v2-1 (Fig. 3).

The voltage-dependence of activation and inactivation of the recombinant channels were determined (Table 1). Most recombinant channels were not different from their parental channels in these gating properties, except for BgNa_v2-1^{A1356V}, BgNa_v2-1^{KT}, and BgNa_v1-1^{V1356A}. BgNa_v2-1^{A1356V} and BgNa_v2-1^{KT} shifted the voltage-dependence of activation by 10 and 9 mV, respectively, in the hyperpolarizing direction. The KT double mutation also caused a 5-mV negative shift in inactivation. In the BgNa_v1-1 background, the reciprocal change, V1356A caused a 4-mV depolarizing shift in the voltage-dependence of activation, suggesting that the background sequence differences in BgNa_v1-1 and BgNa_v2-1 influence the effects of V1356A and A1356V on gating properties.

In conclusion, we demonstrate here that V1356A plays a major role in exon G-mediated different sensitivity of two natural splice variants of the cockroach sodium channel, BgNa_v1-1 and BgNa_v2-1, to deltamethrin and permethrin. The exact mechanism by which V1356A modulates pyrethroid sensitivity remains to be elucidated. The location of V1356A is different from those of previously identified *kdr* and *super-kdr* mutations, which reside within IIS6, IIS5, IIS6, IIS6, the first inter-domain linker, or the inter-cellular loop connecting S4-S5 (Soderlund and Knipple, 2003). It has been predicted that pyrethroid-interacting residues defined by *kdr* mutations may form a pyrethroid binding site(s) near the S6 transmembrane segments in the sodium channel (Lee and Soderlund, 2001; Liu et al., 2002). Recently, we demonstrated that L993F in IIS6 and F1519I in IIS6 indeed reduced the pyrethroid binding to the cockroach sodium channel, revealing the molecular determinants of the pyrethroid receptor on the sodium channel (Tan et al., 2005). Because V1356A is located in the extracellular side of IIS3, outside of the predicted pyrethroid site, its effect on sodium channel sensitivity to pyrethroids may not be exerted through an alteration of pyrethroid binding. There are three alternative mechanisms by which the V1356A change could affect pyrethroid sensitivity. First, the A1356V change could stabilize the open state of the sodium channel, which is more favorable for pyrethroid binding and action (Vais et al., 2000, 2003). Consistent with this mechanism, the BgNa_v2-1^{A1356V} channel had a 10-mV negative shift of voltage-dependence for activation, compared with the

BgNa_v2-1 channel (Table 1). The negative shift in the voltage-dependence of activation could stabilize the open state of the sodium channel and cause the BgNa_v2-1^{A1356V} channel to be more sensitive to pyrethroids. However, BgNa_v2-1^{KT} also shifted the voltage-dependence of activation by 8 mV, but did not alter sodium channel sensitivity to deltamethrin, suggesting that a negative shift in activation gating alone does not always result in enhanced channel sensitivity to deltamethrin. Second, A1356V could affect other gating properties such as deactivation kinetics. It has been reported that neutralization of the fourth positively charged residue in IS4 or IIS4 caused positive shifts in voltage-dependence of both activation and deactivation kinetics (Kontis et al., 1997), suggesting that residues affecting activation may also alter deactivation. Enhanced deactivation by A1356V could counteract the action of pyrethroids which inhibits deactivation. Finally, V1356A may impair the pyrethroid-induced conformational change that is required for pyrethroid action (Table 2). Future research will resolve these possibilities.

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Fig. 1.

A. Topology of the cockroach sodium channel protein, showing the locations and amino acid sequences of exons G1/G2. The alignment of amino acid sequences encoded by exons G1 and G2 are presented above the topology diagram. The residues in G2 that are identical to those in G1 are marked in dots. Single amino acid substitutions (L1346V and A1356V in BgNa_v2-1, boxed), double mutations (LA → FV, IW → SL, RS → KT in BgNa_v2-1, underlined), and triple mutations (GGQ → ADP and MMQ → VWE in BgNa_v1-1, double-underlined) were constructed. The position of A1356V in IIIS3 is indicated with a circle. (B and C). Effects of mutations on channel sensitivity to deltamethrin in the BgNa_v1-1 (B) or BgNa_v2-1 (C) background. Percentages of channels modified by deltamethrin were calculated using the equation $M = \{ [I_{tail}/(E_h - E_{Na})] / [I_{Na}/(E_t - E_{Na})] \} 100$ (Tatebayashi and Narahashi, 1994), as described in Materials and Methods. * indicates a significant difference compared to their parental channels ($p < 0.05$).

Fig. 2. Effects of deltamethrin (a type II pyrethroid) on wild-type and mutant BgNav channels. Deltamethrin-induced tail currents in *Xenopus* oocytes expressing (A) BgNav2-1, (B) BgNav2-1^{A1356V}, (C) BgNav1-1, and (D) BgNav1-1^{V1356A}. (E) Dose–response curves. The percentages of channel modification by various concentrations of deltamethrin were fitted to the Hill equation: $M = M_{\max} / \{1 + (EC_{50} / [\text{deltamethrin}]^n)\}$, as described in Materials and Methods. EC₂₀s values (insert) are derived from the fitted curves.

Fig. 3. Effects of permethrin (a type I pyrethroid) on wild-type and mutant BgNav channels. Permethrin-induced tail currents in *Xenopus* oocytes expressing (A) BgNav₂-1, (B) BgNav₂-1^{A1356V}, (C) BgNav₁-1, and (D) BgNav₁-1^{V1356A}. (E) Dose–response curves. The percentages of channel modification by various concentrations of permethrin were fitted to the Hill equation: $M = M_{\max} / \{1 + (EC_{50}/[\text{permethrin}])^n\}$, as described in Materials and Methods.

Gating properties of recombinant channels in comparison with parental BgNa_v1-1 and BgNa_v2-1 channels

Table 1

Na ⁺ channel type	Activation		Inactivation		n
	V _{1/2} (mV)	K (mV)	V _{1/2} (mV)	K (mV)	
BgNa _v 2-1	-19.2±0.6	4.2±0.1	-45.6±0.6	5.4±0.3	12
FV	-24.9±0.9	3.8±0.6	-51.2±0.7	5.2±0.1	8
SL	-20.0±0.5	4.6±0.6	-47.6±0.6	4.9±0.1	7
KT	-28.5±0.6	4.3±0.5	-51.9±0.4	4.9±0.1	8
L1346V	-18.2±0.9	4.2±0.5	-48.7±0.8	4.7±0.2	10
A1356V	-29.4±0.8	3.8±0.6	-46.0±0.6	4.8±0.3	13
L1346V + A1356V	-25.9±1.0	3.3±0.3	-46.9±0.1	4.5±0.1	8
BgNa _v 1-1	-24.1±0.7	5.5±0.3	-43.5±0.7	5.1±0.2	13
ADP	-24.1±0.3	5.5±0.3	-45.1±0.8	5.9±0.2	6
VWE	-26.1±0.7	5.8±0.4	-47.2±0.6	5.8±0.3	6
V1356A	-20.4±1.0	5.4±0.4	-45.1±0.9	4.8±0.1	8

Table 2

Time constants of the decay of pyrethroid-induced tail currents

Na ⁺ channel type	Deltamethrin ^a		Permethrin ^b	
	τ_1 (s)	τ_2 (s)	τ (s)	<i>n</i>
BgNa _v 1-1	1.56±0.41	0.35±0.08	0.50±0.18	15
BgNa _v 1-1 ^{V1356A}	1.41±0.12	0.31±0.05	0.46±0.13	10
BgNa _v 2-1 ^{A1356V}	1.20±0.18	0.28±0.05	0.25±0.07	8
BgNa _v 2-1	0.88±0.17		0.21±0.06	16

n: Number of oocytes. Time constants of the decay of deltamethrin or permethrin induced tail currents in BgNa_v1-1^{V1356A} and BgNa_v2-1^{A1356V} channel were not significantly different from the parental channels, BgNa_v1-1 and BgNa_v2-1 (*p*<0.05).

^a Deltamethrin-induced tail currents were fitted with double exponential equations except for tail currents of BgNa_v2-1 which were fitted with a single exponential equation. Each value represents the mean ±SEM.

^b Permethrin-induced tail currents were fitted with single exponentials.