A Mutation in the Intracellular Loop III/IV of Mosquito Sodium Channel Synergizes the Effect of Mutations in Helix IIS6 on Pyrethroid Resistance

Lingxin Wang, Yoshiko Nomura, Yuzhe Du, Nannan Liu, Boris S. Zhorov, and Ke Dong

Department of Entomology, Genetics and Neuroscience Programs, Michigan State University, East Lansing, Michigan (L.W., Y.N., Y.D., K.D.); Department of Entomology and Plant Pathology; Auburn University, Auburn, Alabama (N.L.); Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada (B.S.Z.); and Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia (B.S.Z.)

Received July 10, 2014; accepted December 18, 2014

ABSTRACT

Activation and inactivation of voltage-gated sodium channels are critical for proper electrical signaling in excitable cells. Pyrethroid insecticides promote activation and inhibit inactivation of sodium channels, resulting in prolonged opening of sodium channels. They preferably bind to the open state of the sodium channel by interacting with two distinct receptor sites, pyrethroid receptor sites PyR1 and PyR2, formed by the interfaces of domains II/III and I/II, respectively. Specific mutations in PyR1 or PyR2 confer pyrethroid resistance in various arthropod pests and disease vectors. Recently, a unique mutation, N1575Y, in the cytoplasmic loop linking domains III and IV (LIII/IV) was found to coexist with a PyR2 mutation, L1014F in IIS6, in pyrethroid-resistant populations of Anopheles gambiae. To examine the role of this mutation in pyrethroid resistance, N1575Y alone or N1575Y + L1014F were introduced into an Aeles aegypti sodium channel, AaNa+,1–1, and the mutants were functionally examined in Xenopus oocytes. N1575Y did not alter AaNa+,1–1 sensitivity to pyrethroids. However, the N1575Y + L1014F double mutant was more resistant to pyrethroids than the L1014F mutant channel. Further mutational analysis showed that N1575Y could also synergize the effect of L1014S/W, but not L1014G or other pyrethroid-resistant mutations in IIS6 or IS6. Computer modeling predicts that N1575Y allosterically alters PyR2 via a small shift of IIS6. Our findings provide the molecular basis for the coexistence of N1575Y with L1014F in pyrethroid resistance, and suggest an allosteric interaction between IIS6 and LIII/IV in the sodium channel.

Introduction

Voltage-gated sodium channels are responsible for the rapidly rising phase of action potentials (Catterall, 2012). Because of their critical role in membrane excitability, sodium channels are the primary target site of a variety of naturally occurring and synthetic neurotoxins, including pyrethroid insecticides (Catterall et al., 2007). Pyrethroids promote activation and inhibit inactivation of sodium channels, resulting in prolonged opening of sodium channels (Vijverberg et al., 1982; Narahashi, 1996). Pyrethroid insecticides possess high insecticidal activities and low mammalian toxicity and represent one of the most powerful weapons in the global fight against malaria and other arthropod-borne human diseases. However, the efficacy of pyrethroids is undermined as a result of emerging pyrethroid resistance in arthropod pests and disease vectors. One major resistance mechanism is known as knockdown resistance (kdr), which arises from mutations in the sodium channel (Soderlund, 2005; Rinkevich et al., 2013; Dong et al., 2014).

The pore-forming a-subunit of the sodium channel is composed by four homologous domains (I–IV), each having six transmembrane segments (S1–S6) connected by intracellular and extracellular loops. The S1–S4 segments in each domain serve as the voltage-sensing module, whereas the S5 and S6 segments and the loops connecting them function as the pore-forming module. In response to membrane depolarization, the S4 segments move outward, initiating conformational changes that lead to pore opening and subsequent inactivation of sodium channels. Short intracellular linkers connecting S4 and S5 segments of sodium channels, L45, transmit the movements of the voltage-sensing modules to the S6 segments during channel opening and closing. Fast inactivation is achieved by the movement of an inactivation gate formed mainly by the IFM motif in the short intracellular linker connecting domains III and IV, which physically occludes the open pore.

In recent years, using X-ray structures of a bacterial potassium channel KcsA (Doyle et al., 1998), the mammalian voltage-gated potassium channel Kv1.2 crystallized in the open state (Long et al., 2005) and a bacterial sodium channel, AaNav1-1, and the mutants were functionally examined in Xenopus oocytes. N1575Y did not alter AaNav1-1 sensitivity to pyrethroids. However, the N1575Y + L1014F double mutant was more resistant to pyrethroids than the L1014F mutant channel. Further mutational analysis showed that N1575Y could also synergize the effect of L1014S/W, but not L1014G or other pyrethroid-resistant mutations in IIS6 or IS6. Computer modeling predicts that N1575Y allosterically alters PyR2 via a small shift of IIS6. Our findings provide the molecular basis for the coexistence of N1575Y with L1014F in pyrethroid resistance, and suggest an allosteric interaction between IIS6 and LIII/IV in the sodium channel.

This study was supported by the National Institutes of Health National Institute of General Medicine [Grant R01-GM057440]; the National Institutes of Health National Institute of Allergy and Infectious Diseases [Grant R21AI090030]; and the Natural Sciences and Engineering Research Council of Canada [RGPIN-2014-04984].

dx.doi.org/10.1124/mol.114.094730.

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: AaNa+, mosquito sodium channel; kdr, knockdown resistance; LIII/IV, cytoplasmic loop linking domains III and IV; PyR, pyrethroid receptor site.
Na\textsubscript{Ab}, crystallized in the closed state (Payandeh et al., 2011) as templates, homology models of eukaryotic sodium channels have been developed to predict binding sites of sodium channel neurotoxins (Lipkind and Fozzard, 2005; O’Reilly et al., 2006; Tikhonov and Zhorov, 2007, 2012; Du et al., 2013). Mutational analyses coupled with computer modeling show that pyrethroids bind to two analogous receptor sites. Pyrethroid receptor site 1 (PyR1) is formed by residues from helices III\textsubscript{L}45, III\textsubscript{S}, and III\textsubscript{S6} (O’Reilly et al., 2006), whereas pyrethroid receptor site 2 (PyR2) is formed by residues from helices IL\textsubscript{45}, IS\textsubscript{5}, IS\textsubscript{6}, and IS\textsubscript{6} (Du et al., 2013). Binding of pyrethroid molecules at the two sites is believed to effectively trap the sodium channel in the open state, resulting in the prolonged opening of sodium channels (Du et al., 2013).

The most frequent \textit{kd}r mutation in arthropod pests and disease vectors is a leucine to phenylalanine (L\textsubscript{1014}F in the house fly sodium channel) in II\textsubscript{S6}, which is also known as L\textsubscript{216}F using the nomenclature that is universal for sodium channels and other P-loop ion channels (Zhorov and Tikhonov, 2004; Du et al., 2013) (Fig. 1). The L\textsubscript{216}F/S/C/W mutation has been detected in the malaria vector \textit{Anopheles} mosquito species in many regions around the world (Martinez-Torres et al., 1998; Enayati et al., 2003; Karunaratne et al., 2007). Recently, a new sodium channel mutation N\textsubscript{1575}Y was reported in the malaria mosquito, \textit{An. gambiae}, in Africa (Jones et al., 2012). The N\textsubscript{1575}Y mutation is located in the intracellular cytoplasmic loop connecting domains III and IV (LIIL/IV) (Fig. 1). Intriguingly, the N\textsubscript{1575}Y mutation was only found in conjunction with L\textsubscript{216}F: no mosquito individuals were detected harboring only the N\textsubscript{1575}Y mutation (Jones et al., 2012). Interestingly, pyrethroid bioassays indicate that mosquitoes carrying the double mutations L\textsubscript{216}F/S/C/W + N\textsubscript{1575}Y are more resistant to permethrin than mosquitoes carrying only the L\textsubscript{216}F mutation (Jones et al., 2012). However, whether the N\textsubscript{1575}Y mutation confers pyrethroid resistance has not been functionally confirmed yet. In this study, we conducted site-directed mutagenesis, functional analysis in \textit{Xenopus} oocytes, and computer modeling to investigate the role of N\textsubscript{1575}Y in pyrethroid resistance.

**Materials and Methods**

**Site-Directed Mutagenesis.** Because sodium channels from \textit{An. gambiae} have not been successfully expressed in the \textit{Xenopus} oocyte expression system for functional characterization, we used a mosquito sodium channel (AaNa\textsubscript{1-1}), from \textit{Aedes aegypti} to generate all mutants used in this study. The kdr mutations that are explored in this study are located in regions that are highly conserved between sodium channels from \textit{An. gambiae} and \textit{Ae. aegypti} (Supplemental Fig. 1). Site-directed mutagenesis was performed by polymerase chain reaction using Phu Turbo DNA polymerase (Stratagene, La Jolla, CA). All mutagenesis results were confirmed by DNA sequencing.

**Expression of AaNa\textsubscript{1}, Sodium Channels in \textit{Xenopus} Oocytes.** The procedures for oocyte preparation and cRNA injection are identical to those described previously (Tan et al., 2002b). For robust expression of AaNa\textsubscript{1-1} sodium channels, cRNAs were co.injected into oocytes with \textit{Ae. aegypti} Tip\textsubscript{gE} cRNA (1:1 ratio), which enhances the expression of sodium channels in oocytes.

**Electrophysiological Recording and Analysis.** The voltage dependence of activation and inactivation was measured using the two-electrode voltage clamp technique. Methods for two-electrode recording and data analysis were identical to those described previously (Tan et al., 2002a).

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\textsubscript{rev}), where V is the test potential and V\textsubscript{rev} is the reversal potential for sodium ions. Peak conductance values were normalized to the maximal peak conductance (G\textsubscript{max}) and fitted with a two-state Boltzmann equation of the form:

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

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

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

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

in which I is the peak sodium current, I\textsubscript{max} is the maximal current evoked, V is the potential of the voltage prepulse, V\textsubscript{1/2} is the half-maximal voltage for inactivation, and k is the slope factor. Sodium current decay was analyzed by fitting the decaying part of current traces with a single exponential function.

**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., 2002a). The effects of pyrethroids were measured 10 minutes after their application. The pyrethroid-induced tail current was recorded during a 100 pulse train of 5 milliseconds step depolarizations from −120 to 0 mV with 5 milliseconds interpulse interval (Vais et al., 2000). The percentage of channels modified by pyrethroids was calculated using the following equation (see Tatebayashi and Narahashi, 1994):

![Fig. 1. The topology of the sodium channel protein indicating the position of L\textsubscript{216}F/S/C/W and N\textsubscript{1575}Y mutations. The sodium channel protein consists of four homologous domains (I–IV), each formed by six transmembrane segments (S1–S6) connected by intracellular and extracellular loops. Residue positions L\textsubscript{216}F and N\textsubscript{1575}Y correspond to the housefly sodium channel (GenBank accession numbers: AAB47804 and AAB47805). L\textsubscript{216}F is labeled using the nomenclature that is universal for P-loop ion channels, in which a residue is labeled by the domain number (1–4), segment type (\textit{k}, L45 linker; \textit{i}, inner helix, i.e., S6; \textit{o}, outer helix, i.e., S5), and the relative number of the residue in the segment.](image-url)
where $I_{\text{tail}}$ 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.

### Chemicals
Deltamethrin was kindly provided by Bhupinder Khambay ( Rothamsted Research, Harpenden, United Kingdom). Deltamethrin was dissolved in dimethylsulfoxide. The working concentration was prepared in ND96 recording solution immediately prior to experiments. The concentration of dimethylsulfoxide in the final solution was <0.5%, which had no effect on the function of sodium channels.

### Statistical Analysis
Results are reported as mean ± S.E.M. Statistical significance was determined by using one-way analysis of variance with Scheffe’s post hoc analysis, and significant values were set at $P < 0.05$.

## Results

**N1575Y Does Not Alter the Gating Properties of AaNa1-1 Channels.** The N1575Y mutation is located in LiII/IV, which is important for fast inactivation of sodium channels (Catterall, 2002; Goldin, 2003). To determine whether the N1575Y mutation alters inactivation kinetics, we introduced this mutation into AaNa1-1 and expressed both AaNa1-1 and mutant channels in *Xenopus* oocytes. As with AaNa1-1, the mutant channel produced sufficient sodium currents for functional analysis (Fig. 2). AaNa1-1 and N1575Y channels had similar rates of sodium current decay over the entire range of voltages examined, indicating that the mutation did not alter the kinetics of fast inactivation (Fig. 2). Furthermore, the mutation did not alter the voltage dependence of either activation or inactivation (Table 1).

Because the N1575Y mutation coexists with the L1014F mutation in *An. gambiae* (Jones et al., 2012), the N1575Y change was also introduced into AaNa1-1 carrying the L1014F mutation, which was generated in a previous study (Du et al., 2013), to create a double mutation construct L1014F + N1575Y. Similarly, L1014F and L1014F/F + N1575Y channels exhibited similar fast inactivation kinetics compared with AaNa1-1 (Fig. 2). The voltage dependences of activation and inactivation of both mutant channels were also similar to those of the AaNa1-1 channel (Table 1).

**N1575Y Enhances L1014F-Mediated Resistance to Pyrethroids, but Does Not Confer Pyrethroid Resistance Alone.** To examine the effect of the N1575Y mutation on the sensitivity of AaNa1-1 channels to pyrethroids, we compared the sensitivities of AaNa1-1, N1575Y, L1014F, and L1014F/F + N1575Y channels to both a type I pyrethroid, permethrin, and a type II pyrethroid, deltamethrin. The percentage of sodium channel modification by pyrethroids was determined by measuring pyrethroid-induced tail currents upon repolarization in voltage-clamp experiments. The effect of permethrin on the N1575Y channel was similar to that on AaNa1-1 (Fig. 3, A and B). However, the permethrin-induced tail current was reduced by the L1014F/F mutation and more drastically by L1014F/F + N1575Y double mutations (Fig. 3, B–D). Furthermore, analysis of the dose-response curves of modification of AaNa1-1 and the three mutant channels by permethrin and deltamethrin showed that the L1014F/F mutant channel was about 8-fold more resistant to permethrin than the AaNa1-1 channel (Fig. 3E) and the L1014F/F channel was 14-fold more resistant to deltamethrin than the AaNa1-1 channel (Fig. 3F). Remarkably, the L1014F/F + N1575Y channel was 80-fold more resistant to permethrin and 53-fold more resistant to deltamethrin than the wild-type channel. Therefore, the N1575Y mutation increased resistance to permethrin and deltamethrin by 9.8- and 3.4-fold, respectively, when compared with the L1014F/F + N1575Y mutation. However, the N1575Y mutation had no effect on channel sensitivity to either pyrethroid.

**N1575Y Enhances Pyrethroid Resistance Caused by L1014F/W Mutations.** Our aforementioned results indicate that the N1575Y mutation imposes a synergistic effect on pyrethroid resistance caused by the L1014F/F mutation. To see whether such synergism extends to pyrethroid resistance...
Our findings indicate that the $L_{1014}F+N_{1575}Y$ double mutation, which has been reported in resistant mosquitoes, reduces the sensitivity of AaNav1-1 channels to permethrin and deltamethrin. This finding is consistent with previous studies that have shown a significant impact of $kdr$ mutations on the sensitivity of these channels to pyrethroids.

**Figure 3.** The $L_{1014}F+N_{1575}Y$ double mutation reduced the sensitivity of AaNav1-1 channels to permethrin and deltamethrin. (A–D) Tail currents induced by permethrin (1 μM) in AaNav1-1 (A), $N_{1575}Y$ (B), $L_{1014}F$ (C), and $L_{1014}F+N_{1575}Y$ (D) channels. (E and F) Percentage of channel modification by permethrin (E) and deltamethrin (F) of wild-type and mutant channels. Percentage of channels modified by pyrethroids was calculated as described in the Materials and Methods. The values of EC$_{20}$ for permethrin were 0.12, 0.18, 1.0, and 9.8 μM for AaNav1-1, $N_{1575}Y$, $L_{1014}F$, and $L_{1014}F+N_{1575}Y$ channels, respectively. The values of EC$_{20}$ for deltamethrin were 0.1, 0.11, 1.4, and 5.3 μM for AaNav1-1, $N_{1575}Y$, $L_{1014}F$, and $L_{1014}F+N_{1575}Y$ channels, respectively. The number of oocytes for each mutant construct was >5. Each data point indicates mean ± S.E.M. Asterisks indicate significant differences from the AaNav1-1 channel as determined by using one-way analysis of variance with Scheffe’s post hoc analysis, and significant values were set at $P < 0.05$. 

**Table 1.** Voltage dependence of activation and fast inactivation of AaNav1-1 and its mutants.

<table>
<thead>
<tr>
<th></th>
<th>Activation</th>
<th>Fast Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{1/2}$</td>
<td>$k$</td>
</tr>
<tr>
<td>AaNav1-1</td>
<td>$-33.6 \pm 1.1$</td>
<td>$5.7 \pm 0.3$</td>
</tr>
<tr>
<td>$N_{1575}Y$</td>
<td>$-32.3 \pm 1.6$</td>
<td>$6.6 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F$</td>
<td>$-31.3 \pm 0.9$</td>
<td>$4.0 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+N_{1575}Y$</td>
<td>$-26.6 \pm 1.1$</td>
<td>$5.6 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+S$</td>
<td>$-36.3 \pm 1.1$</td>
<td>$4.3 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+S+N_{1575}Y$</td>
<td>$-33.8 \pm 1.2$</td>
<td>$5.1 \pm 0.4$</td>
</tr>
<tr>
<td>$L_{1014}F+W$</td>
<td>$-30.2 \pm 1.1$</td>
<td>$5.0 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+W+N_{1575}Y$</td>
<td>$-29.8 \pm 1.2$</td>
<td>$5.2 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+G$</td>
<td>$-31.5 \pm 1.0$</td>
<td>$4.9 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+G+N_{1575}Y$</td>
<td>$-34.2 \pm 1.2$</td>
<td>$5.0 \pm 0.4$</td>
</tr>
<tr>
<td>$L_{1014}F+N_{1575}Y$</td>
<td>$-37.5 \pm 1.1$</td>
<td>$6.2 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+W$</td>
<td>$-33.0 \pm 1.0$</td>
<td>$5.3 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+W+N_{1575}Y$</td>
<td>$-35.4 \pm 1.1$</td>
<td>$5.6 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+G$</td>
<td>$-30.1 \pm 1.0$</td>
<td>$4.8 \pm 0.5$</td>
</tr>
<tr>
<td>$L_{1014}F+G+N_{1575}Y$</td>
<td>$-32.5 \pm 1.2$</td>
<td>$4.9 \pm 0.4$</td>
</tr>
<tr>
<td>$L_{1014}F+G+N_{1575}Y$</td>
<td>$-29.7 \pm 1.0$</td>
<td>$3.9 \pm 0.1$</td>
</tr>
<tr>
<td>$L_{1014}F+N_{1575}Y$</td>
<td>$-30.7 \pm 0.5$</td>
<td>$4.0 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+W$</td>
<td>$-29.0 \pm 1.1$</td>
<td>$4.2 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+W+N_{1575}Y$</td>
<td>$-28.2 \pm 0.6$</td>
<td>$4.8 \pm 0.2$</td>
</tr>
<tr>
<td>$L_{1014}F+N_{1575}Y$</td>
<td>$-33.2 \pm 1.0$</td>
<td>$5.2 \pm 0.3$</td>
</tr>
<tr>
<td>$L_{1014}F+N_{1575}Y$</td>
<td>$-32.0 \pm 1.2$</td>
<td>$5.0 \pm 0.2$</td>
</tr>
</tbody>
</table>
Tan et al., 2012; Wang et al., 2012). These mutations did not affect the voltage dependence of activation or inactivation of AaNav1-1 channels either alone or in conjunction with the N1575Y mutation (Table 1). Consistent with results from a previous study (Du et al., 2013), the L2i16(1014)S channel was 6.7- and 9-fold more resistant to permethrin and deltamethrin, respectively, than the AaNav1-1 channel (Fig. 4, A and B). The L2i16(1014)S + N1575Y double mutant channel was 58- and 32-fold more resistant to permethrin and deltamethrin, respectively, than the AaNav1-1 channel (Fig. 4, A and B). Similarly, the L2i16(1014)W mutant channel was more resistant to permethrin and deltamethrin than the AaNav1-1 channel by about 9.7- and 7-fold, respectively (Fig. 4, C and D), and introduction of N1575Y mutation into the L2i16(1014)W channel caused additional 10.5- and 13-fold resistance to permethrin and deltamethrin. However, the double mutation L2i16(1014)S + N1575Y double mutant did not produce sufficient sodium currents in oocytes for further functional analysis.

N1575Y Did Not Enhance Pyrethroid Resistance Caused by Another kdr Mutation in IIS6. Next, we examined another kdr mutation in IIS6, V2i18(1016)G, which is detected in pyrethroid-resistant populations of Ae. aegypti (Saavedra-Rodriguez et al., 2007). Although all located in IIS6, L2i16(1014)F/S/W and V2i18(1016)G belong to PyR2 and PyR1, respectively (Du et al., 2013). We introduced the N1575Y mutation into the V2i18(1016)G channel construct, which was available from a previous study (Du et al., 2013). The side chain of L2i16(1014) is directed toward the pyrethroid-sensing residue L1i18 in IS6 (Fig. 6A). Consistent with previous findings (Du et al., 2013), L1i18G reduced AaNav1-1 channel sensitivity to permethrin and deltamethrin. However, the double mutation L1i18G + N1575Y was not more resistant to pyrethroids than the single mutant, L1i18G or S1i29A (Fig. 5, B and C). Furthermore, we introduced two additional substitutions, L1i18F and L1i18W, into both the AaNav1-1 and N1575Y channels. As with L1i18G, both substitutions reduced the action of pyrethroids (Fig. 5, B and C) and the N1575Y mutation did not enhance pyrethroid resistance caused by either mutation (Fig. 5, B and C). In addition, S1i29A at the C end of IS6 is predicted to approach the α-cyano group of deltamethrin and favorably contribute to ligand-channel interactions (Fig. 6, A and B). Here, we showed that S1i29A decreased channel sensitivity to pyrethroids (Fig. 5, B and C), further supporting the PyR2 model. However, the S1i29A + N1575Y channel was not more resistant to both pyrethroids than the single S1i29A mutant. Collectively, these results suggest that the modification of the receptor site by N1575Y may be L2i16(1014) specific. It is possible that unfavorable interactions of the hydrophobic ligand with large aromatic substitutions L2i16(1014)F/W or hydrophilic substitution L2i16(1014)S

Fig. 4. N1575Y enhanced resistance to pyrethroids caused by L1014S/W. Effects of L1014S/W (A, B and C, D, respectively) mutations on the sensitivity of AaNav1-1 channels to permethrin (A and C) and deltamethrin (B and D). Percentages of channel modification by permethrin or deltamethrin were determined using the method described in the Materials and Methods. The number of oocytes for each mutant construct was >5. Each data point indicates mean ± S.E.M. Asterisks indicate significant differences from the AaNav1-1 channel as determined by using one-way analysis of variance with Scheffé’s post hoc analysis, and significant values were set at P < 0.05.
models are provided in the Supplemental Material. In the wild-type channel, deltamethrin favorably interacts with the long flexible side chain of L216(F1014) (Fig. 6A). Comparison of deltamethrin binding models in the wild-type channel and mutants L1118(G (Fig. 6C) and L216(G (Fig. 6D) suggests two consequences of the glycine substitutions. First, the favorable interactions of the hydrophobic ligand with the large hydrophobic pyrethroid-sensing residues are lost. Second, there is significantly more space in the pyrethroid binding site following mutation of L216 to G. Models of deltamethrin binding in the L216S and L216F mutants show unfavorable interactions between the hydrophobic S216 and the large and inflexible F216 with the hydrophobic ligand (Fig. 6, E and F). Therefore, we suggest that enhancement of pyrethroid resistance in the presence of the L216(F1014)F/S/W mutations occurs as a result of a slight shift of IIS6. The shift does not affect either the favorable interaction of pyrethroids with L216 or the less sterically constrained interactions of deltamethrin with the L216(G mutation. In contrast, the slight shift of IIS6 likely further deteriorates the energetically unfavorable interactions of deltamethrin with L216(F1014)F/S/W, enhancing pyrethroid resistance in the double mutant.

**Discussion**

Identification of naturally occurring mutations in the sodium channel that confer kdr to pyrethroids has greatly advanced our understanding of the molecular mechanisms of kdr and the molecular details of pyrethroid receptor sites (Dong et al., 2014). Emerging evidence suggests that binding of pyrethroids to two distinct pyrethroid receptor sites, PyR1 and PyR2, at two analogous domain interfaces is necessary to trap sodium channels in the open state, which leads to prolonged opening of sodium channels and the toxic effects of pyrethroids in vivo. Importantly, many, but not all, kdr mutations that cause pyrethroid resistance are located within the two pyrethroid receptor sites (Dong et al., 2014), and it is not clear how kdr mutations beyond the receptor sites affect pyrethroid sensitivity of sodium channels. In this study, we investigated the role of a pyrethroid resistance-associated mutation, N1575Y, which is located in the intracellular loop connecting domains III and IV (outside of PyR1 and PyR2) in pyrethroid resistance. We found that this mutation alone has no effect on the action of pyrethroids on sodium channels, but it enhances pyrethroid resistance caused by the L216(F1014)F/S/W mutations. Based on further mutational analysis and computer modeling, we hypothesize that N1575 induces a small shift of the transmembrane helix IIIS6, resulting in slight deformation of PyR2, which enhances the energetically unfavorable interactions between deltamethrin and the L216(F1014)F/S/W mutations, but does not affect the energetically favorable interactions between pyrethroids and L216(F1014). These results provide a satisfying explanation for the concurrent existence of N1575Y and L216(F1014)F in pyrethroid-resistant mosquito populations.

BLAST searches show that the asparagine residue (equivalent to N1575 in An. gambiae) in the LIII/IV loop sequence is highly conserved among voltage-gated sodium channels. However, N1575Y has no effect on the kinetics of fast inactivation, implying that this mutation does not interfere with the docking of the inactivation particle (the motif MFMT in LIII/IV) to its receptor site, which is composed of residues in the linkers connecting S4 and S5 of domains III and IV (Goldin, 2003). Our
molecular modeling (Fig. 6) predicts that a slight shift of helix IIS6 in the N1575Y channel could explain the synergism on pyrethroid resistance between N1575Y and specific mutations at L2i16(1014)F in IIS6, which is also supported by our mutational analysis (Figs. 3–5). However, we cannot completely rule out the possibility of the N1575Y/L2i16(1014)Fd double mutation causing an allosteric effect on the action of pyrethroids without directly involving IIS6. How a mutation in the intracellular loop between domains III and IV (LIII/IV) can shift helix IIS6 carrying the L2i16(1014)F mutation remains speculative. In X-ray structures of both open and closed ion channels, the cytoplasmic parts of the S6 helices approach each other to form the activation gate, and cytoplasmic linkers may also approach each other. We speculate that the asparagine side chain in LIII/IV is involved in specific contacts (likely, an H-bond) with LII/III. Replacement of a small asparagine with a much bigger tyrosine in N1575Y could cause a change in the mutual disposition of the two linkers, which, in turn, would shift helix IIS6. This predicted small shift in IIS6 by N1575Y apparently only alters the action of pyrethroids on L2i16(1014)F/S/C/W channels, but not on L2i16G and V2i18(1016)G channels. This may be because a small distortion of PyR2 upon the N1575Y mutation does not affect a weak pyrethroid interaction with a glycine residue, which has a single hydrogen atom in the side chain.

Besides N1575Y, there are several other sodium channel mutations within LIII/IV that have been reported to be associated with pyrethroid resistance (Rinkevich et al., 2013; Dong et al., 2014). The frequent occurrence of mutations associated with pyrethroid resistance in this linker supports the allosteric interactions between IIS6 and LIII/IV in the sodium channel. For example, L1596P was found to be associated with pyrethroid resistance in V. mites (Fig. 5) (Dong et al., 2014). Although this mutation has not been functionally examined using V. mite sodium channels expressed in Xenopus oocytes, insect sodium channels possess...
a proline at the corresponding position and the leucine substitution of proline in the cockroach sodium channel renders the cockroach sodium channel more sensitive to pyrethroids (Liu et al., 2006). Therefore, the L1596P mutation is predicted to make the varroa mite sodium channel more resistant to pyrethroids. Since L1596P alone could confer pyrethroid resistance, P1596, located at the C-terminus of III/IV, likely has a more drastic allosteric effect on pyrethroid binding (to PyR1 and/or PyR2) than N1575Y.

The involvement of other intracellular linkers in pyrethroid resistance has also been reported. E435K and C785R, in the linker connecting domains I and II (LI/II), were identified in pyrethroid resistant German cockroach populations (Liu et al., 2000; Dong et al., 2014). As with N1575Y, E435K or C785R alone did not reduce sodium channel sensitivity to pyrethroids. However, concurrence of either E435K or C785R mutation with the kdr mutations, V1081I and M in IS6 or L1014F in IS5, significantly increases pyrethroid resistance (Liu et al., 2002; Tan et al., 2002b). A mechanism similar to that for N1575Y could explain the role of E435K or C785R in pyrethroid resistance. Another example is G1111 (in the cockroach sodium channel) in the second intracellular linker connecting domains II and III (LI/II), which is selectively involved in the response of sodium channels to type II pyrethroids, such as deltamethrin (Du et al., 2009). Deletion of G1111 (due to alternative splicing of the channel) in the second intracellular linker connecting domains II and III (LI/II), was identified in pyrethroid resistant populations of Sri Lankan malaria vectors. *Pestic Biochem Physiol* 88:118–113.


a novel mutation, V1010L, in the voltage gated Na⁺ channel of *Anopheles culicifacies* from Orissa, India. *Malar J* 9:146.


Address correspondence to: Ke Dong, Department of Entomology, Genetics and Neuroscience Programs, Michigan State University, East Lansing, MI 48824. E-mail: dongk@cns.msu.edu