Rotational Symmetry of Two Pyrethroid Receptor Sites in the Mosquito Sodium Channel

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ABSTRACT

Voltage-gated sodium channels are the primary target of pyrethroid insecticides. Although it is well known that specific mutations in insect sodium channels confer knockdown resistance (kdr) to pyrethroids, the atomic mechanisms of pyrethroid-sodium channel interactions are not clearly understood. Previously, computer modeling and mutational analysis predicted two pyrethroid receptors, pyrethroid receptor site 1 (PyR1) (initial) and pyrethroid receptor site 2 (PyR2), located in the domain interfaces II/III and I/II, respectively. The models differ in ligand orientation and the number of transmembrane helices involved. In this study, we elaborated a revised PyR1 model of the mosquito sodium channel. Computational docking in the Kv1.2-based open channel model yielded a complex in which a pyrethroid (deltamethrin) binds between the linker helix IL45 and transmembrane helices IIS5, IIS6, and IIII6 with its dibromoethynyl and diphenylether moieties oriented in the intra- and extracellular directions, respectively. The PyR2 and revised PyR1 models explained recently discovered kdr mutations and predicted new deltamethrin-channel contacts. Further model-driven mutagenesis identified seven new pyrethroid-sensing residues, three in the revised PyR1 and four in PyR2. Our data support the following conclusions: 1) each pyrethroid receptor is formed by a linker-helix L45 and three transmembrane helices (S5 and two S6s); 2) IIS6 contains four residues that contribute to PyR1 and another four to PyR2; 3) seven pairs of pyrethroid-sensing residues are located in symmetric positions within PyR1 and PyR2; and 4) pyrethroids bind to PyR1 and PyR2 in similar orientations, penetrating deeply into the respective domain interfaces. Our study elaborates the dual pyrethroid-receptor sites concept and provides a structural background for rational development of new insecticides.

Introduction

Pyrethroid insecticides are used extensively for the control of insect pests and disease vectors involved in the transmission of various human diseases, including malaria and dengue (WHO, 2007). Pyrethroids exert toxic effects by altering the gating of voltage-gated sodium channels (Narahashi, 1986, 1996; Bloomquist, 1996; Soderlund, 2010), which are essential for electrical signaling in the nervous system.

Unlike mammalian sodium channels, insect sodium channels comprise four homologous domains (I–IV), each having six membrane spanning helical segments (S1–S6) (Catterall, 2012; Dong et al., 2014). Segments S1–S4 in each domain constitute a voltage-sensing module, which is connected through a linker-helix (L45) to a pore-forming module. The pore module is composed of an outer helix S5, a pore-lining inner helix S6, and a membrane-re-entrant P-loop from each domain. The pore module forms a central pore, whereas the four voltage-sensing modules are arranged around the pore module. In response to membrane depolarization, the S4 segments move outward, initiating opening of the activation gate formed by cytoplasmic parts of S6s (i.e., channel activation).

A major threat to the sustained use of pyrethroids in pest and vector control is the development of pyrethroid resistance. A well known mechanism of pyrethroid resistance, knockdown resistance (kdr), is caused by naturally occurring sodium channel mutations (Soderlund, 2005; Davies et al., 2007; Rinkevich et al., 2013; Dong et al., 2014). The emerging pyrethroid resistance demands development of new insecticides. In the absence of X-ray structures of eukaryotic sodium channels, rational development of new pyrethroids can be facilitated by building homology models of insect sodium channels and computational docking of pyrethroids in these models.

The first pyrethroid receptor site model (O’Reilly et al., 2006) was elaborated for the house fly open sodium channel using the X-ray structure of a voltage-gated potassium channel K1.2 (Long et al., 2005) as a template. According to this model, pyrethroids bind to the lipid-exposed interface formed by IIL45, which connects the second-domain transmembrane helices IIS4 and IIS5, the outer helix IIS5, and the inner helix IIII6.

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ABBREVIATIONS: DMT, deltamethrin; kdr, knockdown resistance; MCM, Monte Carlo minimization; PMT, permethrin; PyR1, pyrethroid receptor site 1; PyR2, pyrethroid receptor site 2.
Kv1.2-based homology model of a mosquito sodium channel
A revised PyR1 model, which includes IIS6. We used our
model toward the PyR1, suggesting that IIS6 be part of PyR1. In
the initial PyR1 model (O

This valine was not indicated as a pyrethroid-sensing residue in
the AaNav1-1 sodium channel to pyrethroids (Du et al., 2013).

stream from the gating-hinge glycine) reduces the sensitivity of
seven moieties oriented in the intra- and extracellular directions,
respectively. In contrast, DMT is predicted to bind in PyR2 in
a reverse orientation, with dibromoethenyl and diphenylether moiety orientations in the intra- and extracellular directions,
respectively.

Earlier we demonstrated that a kdr mutation of valine to
glycine (V1023G) in the middle of IIS6 (four positions
downstream from the gating-hinge glycine) reduces the sensitivity of
the AaNav1-1 sodium channel to pyrethroids (Du et al., 2013).
This valine was not indicated as a pyrethroid-sensing residue in
the initial PyR1 model (O’Reilly et al., 2006), but it is oriented
toward the PyR1, suggesting that IIS6 be part of PyR1. In
this work, we explored this possibility by docking DMT into a
revised PyR1 model, which includes IIS6. We used our
K_1.2-based homology model of a mosquito sodium channel (Du et al., 2013) to simultaneously dock two DMT molecules to the channel. We propose a model of the sodium channel with two DMT molecules in which one ligand binds to PyR2, as we predicted before (Du et al., 2013), whereas another ligand binds quasisymmetrically to the revised PyR1 model, between the linker helix IL45 and transmembrane helices IIS5, IIS6, and IIIIS6. Dibromoethenyl and diphenylether moieties of both DMT molecules are oriented in the intra- and extracellular directions, respectively.

Subsequent model-driven mutagenesis followed by electrophysiological studies unveiled three new pyrethroid-sensing residues in the revised PyR1 model and four in PyR2. The PyR2 and revised PyR1 models display rotational quasi-symmetry around the pore axis and have many common features. Our study provides new insights into the concept of dual pyrethroid receptor sites and forms a structural background for rational development of new pyrethroid insecticides.

Materials and Methods

Computer Modeling

The X-ray structure of the K_1.2 channel (Long et al., 2005) was used as a template to build the open conformations of the AaNa_1-1 channel. Sequence alignment of K_1.2 and AaNa_1-1 channels is shown in Fig. 1. Homology modeling and ligand docking were performed using the ZMM program (www.zmmsoft.com; see Garden and Zhorov, 2010) and Monte Carlo minimization (MCM) protocol (Li and Scheraga, 1987), as described in our previous study (Du et al., 2013). Molecular images were created using the PyMol Molecular Graphics System, version 0.99rc6 (Schrodinger, New York, NY).

Homology models of heterotetrameric asymmetric eukaryotic sodium channels are not expected to be as precise as X-ray structures of homotetrameric symmetric ion channels. Therefore, an apparent global minimum found through unbiased hands-free docking of a highly flexible ligand, like pyrethroids, is unlikely to correspond to the real structure of the ligand-channel complex. A solution to this problem is a biased docking of ligands to ligand-sensing residues, which are known from experiments. The biased docking involves distance constraints between a ligand and ligand-sensing residues. When a specific ligand-channel distance in the model exceeds the upper distance constraint limit (which is usually set to 4–5 Å), a large energy penalty is added to the model energy. An MCM protocol modifies the model geometry and minimizes the energy simultaneously with other energy terms, including van der Waals and electrostatic interactions. The distance constraints are considered to be satisfied when the constraints energy reaches zero. Further MCM steps optimize the ligand-channel interactions. To preclude large deviations of the channel backbones from the X-ray templates (and thus preserve the channel folding), another set of distance constraints (pins) is set between matching α carbons in the template and the model. A pin constraint is a flat-bottom parabolic energy function that allows an atom (in this study, an α carbon) to deviate penalty free of up to 1 Å from the template and imposes a penalty of 10 kcal mol^{-1} Å^{-1} for larger deviations. The pin constraints are necessary because the initial relaxation of an unconstrained homology model with a large ligand would cause significant deviations of the model backbones from the template due to steric clashes between the ligand and the protein.

Mutational data do not reveal specific atom-atom interactions between a ligand and a mutated side chain. Therefore, during ligand docking we used ligand–side chain constraints. Each such constraint specifies a ligand, a residue in the protein, and the targeted distance between the ligand and the residue side chain, which was set to 5 Å. Each constraint instructed the ZMM program to choose a closest pair of atoms between the ligand and specific side chain and apply a distance constraint to these atoms. The closest pairs of atoms are selected in the beginning of each energy minimization, thus allowing

<table>
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<tr>
<th>Channel</th>
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<th>#</th>
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<th>k1l</th>
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<td>SSAYVFAE</td>
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<td>²</td>
<td>RTMGNZND</td>
<td>PVCFIIIF</td>
<td>AWGMLLF</td>
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<tr>
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Fig. 1. Aligned sequences of K_1.2 and AaNa_1-1 channels indicating residues that are predicted to contribute to PyR1 or PyR2 or control ligand access to PyR1 or PyR2. PyR1 residues are highlighted, and PyR2 residues are underlined. Substitutions of these residues have been tested experimentally previously or in this study (Table 1).
modification of atom-atom constraints during the MCM search for the apparent global minimum.

To overcome the problem of different residue numbers in homologous positions of sodium channels in different organisms and to highlight symmetric locations of residues in different channel domains, we used a residue-labeling scheme, which is universal for P-loop channels (Zhorov and Tikhonov, 2004; Du et al., 2013). A residue label includes the domain number (1–4), segment type (k, the linker-helix L45; i, the inner helix S6; and o, the outer helix S5), and relative number of the residue in the segment (see Fig. 1).

Site-Directed Mutagenesis

We used a mosquito sodium channel, AaNa1-1, from Aedes aegypti to generate all mutants used in this study. Site-directed mutagenesis was performed by polymerase chain reaction using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA). All mutagenesis results were confirmed by DNA sequencing.

Expression of AaNa1, Channels in Xenopus Oocytes and Electrophysiology

Procedures for preparation of oocytes and cRNA and injection were identical to those described previously (Tan et al., 2005). Methods and data analysis for two-electrode voltage clamp recording of sodium currents and measurement of tail currents induced by pyrethroids were identical to those previously described (Tatebayashi and Narahashi, 1994; Tan et al., 2005). All experiments were performed at room temperature. Sodium currents were measured with an OC725C oocyte clamp (Warner Instruments, Hamden, CT) and a Digidata 1440A interface (Axon Instruments, Foster City, CA), pCLAMP 10.2 software (Axon Instruments) was used for data acquisition and analysis.

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.

Chemicals. DMT was provided by R. Nauen (Bayer CropScience AG, Monheim, Germany). Permethrin (PMT) was purchased from ChemService (West Chester, PA). Pyrethroids were dissolved in dimethylsulfoxide in a 100 mM stock. The working concentrations were 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.

Results

Docking Two Deltamethrin Molecules in the AaNa1-1 Channel Model. As a starting point, we used our AaNa1-1 model with DMT bound to PyR2 (Du et al., 2013). In this model, the dibromoethenyl and diphenylether moieties of DMT are oriented, respectively, in the intra- and extracellular directions, whereas the bulky, rigid dimethycyclopropane fragment fits between helices IL45, IS5, IS6, and IIS6 (Fig. 2A). We placed the second DMT molecule between helices IIL45, IIS5, IIIS6, and IIS6 (Fig. 2B) and set the ligand starting conformation and orientation as for DMT in PyR2. We further imposed two ligand–side chain distance constraints involving most separated PyR1 residues, L2k7 (Usherwood et al., 2007) and V2i18 (Du et al., 2013), and performed three stages of MCM. In the first stage, the channel backbones and the ligands’ bond angles were kept rigid to avoid their large deformations due to very strong repulsion with the ligand that was manually placed into PyR1. At the second stage, all the torsional and bond angles were allowed to vary, but pins and the ligand–side chain constraints were preserved. This stage yielded a low-energy structure in which the distance constraints were satisfied and both DMT molecules had only attractive (negative-energy) interactions with the channel residues. In the third stage, all the constraints were removed. The energy further decreased, whereas the DMT-channel geometry changed insignificantly. In particular, none of the α carbons deviated more than 1.5 Å from the respective template positions.

The ternary complex of AaNa1-1 with two DMT molecules is shown in Fig. 2, C and D. Both ligands bind into respective domain interfaces and interact with the channel residues, many of which are in symmetric positions (Fig. 1). Orientation of the two DMT molecules, which are bound to PyR2 and revised PyR1 models, is similar, but not identical (Fig. 2, A and B). Both DMT molecules bind deeply in domain interfaces (Fig. 2, C, E, and F), whereas their terminal aromatic rings approach the inner pore without blocking the ion permeation (Fig. 2D). A total of two linker helices (L45) and five transmembrane helices (S5 and S6) contributes to PyR1 and PyR2. Among these only IIS6 is a part of both receptor sites, contributing four residues to PyR1 and six residues to PyR2 (Fig. 2D). One face at the extracellular half of IIS6 contributes to PyR2, whereas the opposite face in the intracellular half of the helix contributes to PyR1 (Fig. 2D). The only exception is the pore-facing residue F2i22 that is close to the terminal aromatic rings of the ligands bound to PyR1 and PyR2 (Fig. 2D). In both ligands, the bulky dimethycyclopropyl moiety fits in the kink region between a linker helix L45 and the outer helix S5. The nitrile group approaches a conserved asparagine in position i20 (S6) and its putative open-state H-bonding partner in position i29 of the preceding domain (Tikhonov et al., 2015). The predictability of our model was tested by mutational analysis, as described below.

Mutational Analysis Confirmed New Pyrethroid-Sensing Residues in PyR1 and PyR2. Our model of the AaNa1-1 sodium channel with DMT molecule bound to PyR1 is consistent with published experimental data, which describe pyrethroid-sensing residues L2k7, M2k11, L2o6, V2i18, F3i13, F3i17, and N3i20 (see Fig. 3; Table 1; and references therein). Among these eight previously known contributors to PyR1, mutations of five residues have been identified in pyrethroid-resistant populations as kdr mutations, as follows: M2k11T, L2o6I, V2i18G, F3i13C, and F3i17F (Guerrero et al., 1997; He et al., 1999; Morin et al., 2002; Brengues et al., 2003; Kawada et al., 2009). Our PyR2 model (Du et al., 2013) described five pyrethroid-sensing residues (I2k7, V2i11, L1i18, L1i19, and L2i16), and intensive MCM of the channel model with two DMT molecules did not reveal any conflict between the ligands bound to PyR1 and PyR2, thus confirming our PyR2 model (Du et al., 2013).

Importantly, the analysis of the channel model with two DMT molecules revealed 10 residues that interact with the bound ligands (Fig. 3), but have not been previously described as components of PyR1 or PyR2. These 10 residues include three residues in PyR1 (F2i22, L2i25, and L2i26) and seven residues in PyR2 (I2k12, L1o6, I1i22, I1i25, S1i29, N2i15, and N2i20). Mutations of three of the 10 residues, S2i15I, E2i22S, and L2i26A, have been previously associated with pyrethroid resistance: N2i15S from Anopheles sinensis (Tan et al., 2012); F2i22S and S1i29S from Blattella germanica (Pridgeon et al., 2002) and Plutella xylostella (Enderby et al., 2011); and L2i26F from Tetranychus urticae (Kwon et al., 2010). We mutated the 10 pyrethroid-sensing residues in PyR1 and PyR2, as well as some residues beyond PyR1 and PyR2 and explored the actions of DMT and PMT on the mutants.
For known kdr mutants, we tested the amino acid substitutions identified in resistant insects (e.g., F2i22S), whereas for new model-predicted pyrethroid-sensing residues we evaluated alanine substitutions.

A total of 17 single mutants and two double mutants of the AaNav1-1 channel was investigated. All mutant channels generated sufficient sodium currents in Xenopus oocytes for further functional analysis. With only a few exceptions, most mutant channels showed insignificant changes in gating properties (voltage dependence of activation and fast inactivation) when compared with wild-type channels (Table 2). However, channels incorporating the L1o6A mutation displayed dramatic hyperpolarizing shifts in the voltage dependence of both activation and inactivation. In addition, mutations I1i25A and L2i26A each caused hyperpolarizing shifts (∼14 and 10 mV, respectively) in the voltage dependence of inactivation in AaNav1-1 channels (Table 2).

To evaluate channel sensitivity to pyrethroids, the percentage of sodium channels modified by DMT or PMT was determined by the method developed by Tatebayashi and Narahashi (Tatebayashi and Narahashi, 1994). Results of these experiments are shown in Fig. 4. We observed strong reduction of sensitivity with mutation of three residues within PyR1 (F2i22S, L2i26A, and L2i26A) and four within PyR2 (S1o2A, I1i25A, S1i29A, and N2i15S) to both DMT and PMT. These results confirm the model-predicted pyrethroid-sensing residues in both PyR1 and PyR2 models. In particular, our experiments confirmed that the revised PyR1 model includes helix IIS6, which was not a part of the initial PyR1 model (O’Reilly et al., 2006).

Our data show generally similar effects of mutations on the action of DMT and PMT (Fig. 4), suggesting that both ligands bind rather similarly to respective receptor sites. It should be noted that mutational analysis cannot demonstrate specific interactions of individual DMT moieties with individual chemical groups of the mutated residues. Therefore, we refrained in this study from docking PMT to PyR1 or PyR2. Our model of the channel with PMT bound to PyR2 is available elsewhere (Du et al., 2013). An approach to address the challenging problem of atomic details of ligand-channel interactions should involve
exploration of action of different pyrethroids on different mutants. This massive task is beyond the goal of our current study.

**Effect of Mutations beyond PyR1 and PyR2.** Residues N\(^{105}\) and R\(^{107}\) are located around the predicted PyR2, but do not interact with pyrethroids in our model. In agreement with the model, mutations N\(^{105}A\) and R\(^{107}A\) had but small effects on the action of pyrethroids (Fig. 4).

Glycine G\(^{119}\) in the IL45 helix is conserved in insect and mammalian sodium channels (Du et al., 2013). In our model, G\(^{119}\) is exposed to the cytoplasm and may interact with a cytoplasmic part of the channel through a knob-into-hole contact. We expressed mutants G\(^{119}A\) and G\(^{119}I\) and found that they substantially decreased the channel sensitivity to pyrethroids (Fig. 4). We further expressed and tested two double mutations (G\(^{119}A\)\(^{119}I\) and G\(^{119}I\)\(^{119}A\)), but did not find any synergistic effects of the mutations on the pyrethroid action (Fig. 4).

**Discussion**

**Common and Unique Features of the Initial and Revised PyR1 Models.** The pioneering PyR1 model for the housefly sodium channel (O'Reilly et al., 2006) and *Drosophila* sodium channel (Usherwood et al., 2007) and the revised PyR1 model elaborated in this study for the AaNav1-1 channel have several common features. These include the location of the receptor site in the II/III domain interface, extended conformation of the receptor-bound DMT, and direct contacts of DMT with seven experimentally determined pyrethroid-sensing residues in helices II\(^{L45}\) (M\(^{211}\)), II\(^{S5}\) (L\(^{210}\), T\(^{210}\)), and II\(^{S6}\) (F\(^{313}\), F\(^{316}\), and F\(^{317}\)). However, there are four main differences between the initial and revised PyR1 models. First, in the initial model, DMT binds at the protein surface and interacts with three helices (II\(^{L45}\), II\(^{S5}\), and II\(^{S6}\)), whereas in our revised model DMT binds deeply in

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**TABLE 1**

<table>
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<tr>
<th>Mutant</th>
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<th>PyR2</th>
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<tr>
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<tr>
<td>N(^{320}A)</td>
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</table>

\(^a\)References: 1 (Usherwood et al., 2007); 2 (Du et al., 2009); 3 (Du et al., 2013) and references therein; 4 (Tan et al., 2005); and 5, this study (Figs. 3B and 4).

\(^b\)F\(^{212}\) contributes to both PyR1 and PyR2.

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**Fig. 3.** Topology of the AaNav1-1a sodium channel. (A) Pyrethroid-sensing residues are indicated for PyR1 (open circles) and PyR2 (gray circles), respectively. Mutations that are detected in pyrethroid-resistant field populations are underlined. (B) Mutations tested in our study. Previously discovered kdr mutations are underlined. Open and solid circles indicate mutations within or nearby PyR1 and PyR2, respectively.
C2o14 is rather far from the ligand. Finally, in the revised PyR1 model (Usherwood et al., 2007), whereas in the revised PyR1 model pyrethroid-sensing residues decrease potency of pyrethroids, were found in the subsequent stages of MCM docking that did biased overall orientation of DMT in PyR1 in only the first PyR2 (Du et al., 2013). The above two distance constraints systematic exploration of different possibilities in modeling system, DMT interacts with L2k7 in the middle part of the IIL45 constraints to direct dibromoethenyl and diphenylether moieties of DMT bound in the initial PyR1 model are oriented in the extra- and intracellular directions, diphenylether moieties of DMT bound in the initial PyR1 (IIL45, IIS5, IIS6, and IIIS6). Second, dibromoethenyl and (I1k12N, I3i12, like V2i12, extends toward T2o10 and both residues may control access of DMT to PyR1 (Fig. 2F). Alanine substitutions of the bulky β-branched residues I3i12 or V2i12 could widen the access path for pyrethroids from the membrane to reach the receptors and thus increase, rather than decrease, the channel sensitivity to pyrethroids. Substitution of the β-branched V2i12 by a bigger, but more flexible leucine may also facilitate the ligand access to PyR2, and our data are consistent with this proposition (Fig. 4). The opposite leafs of the gates that separate PyR1 and PyR2 from lipids contain β-branched T2o10 and I1o10, respectively. We are not aware of any substitutions of these residues, but substitutions T2o10I1 and I1o10C by bigger residues decrease the ligands potency (Table 1), which may be explained in our models of PyR1 and PyR2 by a more restricted access path for pyrethroids from the membrane to their receptors. V2i12A and V2i12L are putative kdr mutations in PyR2, which are found to coexist with kdr mutations M2k11L and L2i16S in pyrethroid-resistant populations of Thrips tabaci (Wu et al., 2014) and Anopheles culicifacies (Singh et al., 2010). Importantly, M2k11L and L2i16S significantly reduce channel sensitivity to pyrethroids (Table 1), and we suggest that the double mutations may have synergistic effects due to decreasing the ligand-channel interactions and facilitating the ligand egress from PyR1 and PyR2. Another residue that may control access of pyrethroids to PyR2 is C2o14. Mutation C2o14 decreases potency of DMT, but increases potency of PMT (Table 1). In our model, C2o14 is rather far from PyR1-bound DMT, but it interacts with T2o10 and thus may indirectly control access of pyrethroids to PyR1 (Fig. 2F).

**TABLE 2**  
Voltage dependence of activation and inactivation of mosquito sodium channels

<table>
<thead>
<tr>
<th>Na⁺ Channel Type</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V1/2 (mV)</td>
<td>k (mV)</td>
</tr>
<tr>
<td>AnNa₃, I-1</td>
<td>-29.2 ± 0.8</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>G13K1A</td>
<td>-32.6 ± 1.2</td>
<td>5.0 ± 0.2</td>
</tr>
<tr>
<td>G13K1</td>
<td>-32.8 ± 1.4</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>I1k12N</td>
<td>-33.8 ± 0.9</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>I1k12N*G13K1A</td>
<td>-30.2 ± 1.0</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>I1k12N*G13K1A</td>
<td>-30.4 ± 0.5</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>S12i2A</td>
<td>-32.9 ± 0.2</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>S12i2A</td>
<td>-33.5 ± 0.8</td>
<td>4.5 ± 0.2</td>
</tr>
<tr>
<td>I1k12A</td>
<td>-50.5 ± 1.8</td>
<td>6.1 ± 0.6</td>
</tr>
<tr>
<td>I1k12A</td>
<td>-31.0 ± 0.6</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>I1k12A</td>
<td>-34.3 ± 1.1</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>I1k12A</td>
<td>-30.0 ± 0.8</td>
<td>7.0 ± 0.6</td>
</tr>
<tr>
<td>S12i2A</td>
<td>-33.0 ± 0.8</td>
<td>5.5 ± 0.4</td>
</tr>
<tr>
<td>V2i12A</td>
<td>-26.7 ± 0.7</td>
<td>5.6 ± 0.4</td>
</tr>
<tr>
<td>V2i12A</td>
<td>-32.7 ± 0.8</td>
<td>5.5 ± 0.3</td>
</tr>
<tr>
<td>N2i12S</td>
<td>-28.8 ± 1.3</td>
<td>5.1 ± 0.1</td>
</tr>
<tr>
<td>N2i12S</td>
<td>-30.8 ± 0.6</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>F2i12S</td>
<td>-30.7 ± 0.9</td>
<td>5.3 ± 0.7</td>
</tr>
<tr>
<td>L2i2A</td>
<td>-28.5 ± 0.8</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>L2i2A</td>
<td>-31.7 ± 1.1</td>
<td>7.2 ± 0.4</td>
</tr>
</tbody>
</table>

Some features of the revised DMT-bound PyR1 model were predetermined by the fact that we imposed two distance constraints to direct dibromoethenyl and diphenylether moieties toward L2k7 and V4i18, respectively. These constraints forced DMT to adopt in PyR1 orientation analogous to that of PyR2-bound DMT whose dibromoethenyl and diphenylether moieties interact with I1k12 and L1i13, respectively. Such orientation of the bound DMT was found optimal in the systematic exploration of different possibilities in modeling PyR2 (Du et al., 2013). The above two distance constraints biased overall orientation of DMT in PyR1 in only the first stage of MCM docking, whereas all specific DMT-PyR1 contacts were found in the subsequent stages of MCM docking that did not involve any ligand-channel distance constraints. It should also be noted that the initial PyR1 model was built based on experimental data available by 2006, whereas our revised PyR1 model was built using experimental data available as of 2014 (Fig. 3).

**Some Mutations within PyR1 and PyR2 Increased the Potency of Pyrethroids.** Usually substitutions of pyrethroid-sensing residues decrease potency of pyrethroids, but there are intriguing exceptions. In our experiments, mutations V2i12A and V2i12L within PyR2 did not decrease potency of either PMT or DMT, but appear to slightly increase potency of both PMT and DMT (Fig. 4; Table 1). In our PyR2 model, V2i12L is exposed toward I1o10 (Fig. 2E), and both residues may control the DMT access to PyR2. In earlier experiments, we found similar effects of increasing DMT and PMT potency in the analogous I3i12A mutation in PyR1 of the cockroach sodium channel (Du et al., 2009). In our revised PyR1 model, I3i12, like V2i12, extends toward T2i10, and both residues may control access of DMT to PyR1 (Fig. 2F).
Rotational Symmetry of PyR1 and PyR2. Figure 2, C and D, illustrates rotational symmetry of PyR1 and PyR2. Thus, clockwise rotation by 90° of the cytoplasmic view (Fig. 2D) would put PyR1 in place of PyR2. To some extent, the symmetric disposition of ligands in PyR1 and PyR2 was imposed due to the biased docking of DMT to L2k7 and V2i18. However, the docking predicted three new residues in PyR1 (F2i22, L2i25, and L2i26), and mutational analysis confirmed these predictions (Fig. 4). The symmetric positions of pyrethroid-sensing residues in PyR1 and PyR2 are also illustrated in Table 1 and Fig. 1. Figure 1 highlights pyrethroid-sensing residues that, according to mutational analysis, contribute to PyR1 (bold and highlighted) and PyR2 (bold and underlined). A total of 11 residues in PyR1 has matches in PyR2 (Table 1), and the majority of residues in these matching positions are hydrophobic, but structurally different amino acids.

Mutational analysis also confirmed several new pyrethroid-sensing residues in PyR2 (Fig. 4). Of course, the symmetry of PyR1 and PyR2 is not ideal due to sequence differences of the four channel repeat domains. For example, we observed a significant decrease in the potency of DMT and PMT in the kdr mutant N2i15S. In our model, N215 is close to the terminal phenyl ring of PyR2-bound DMT (Fig. 2A). Mutation S3i15A in the matching position of PyR1 has only a small effect on the DMT and PMT action (Du et al., 2009). This is consistent with our model in which the small side chain of S3i15 is farther from PyR1-bound DMT than the side chain of N2i15 from the PyR2-bound DMT.

Mutation L2o6I in PyR1 decreases the sensitivity of sodium channels to DMT and PMT, whereas mutations L1o6I/A in the matching position of PyR2 had no effect on pyrethroid sensitivity (Table 1). Our model predicts that L2o6 and L1o6 control the ligand access to PyR1 and PyR2, respectively (Fig. 2, E and F), and also directly interact with the receptor-bound pyrethroids. The combined effects of these two factors may depend on peculiarities of the amino acid substitutions. In addition, mutation L2i25A in PyR1 was very resistant to both DMT and PMT, whereas mutation I1i25A in the matching position of PyR2 had no effect on the action of pyrethroids (Table 1). The cause of this asymmetry is less clear.

In our model, the nitrile groups of DMT molecules approach conserved asparagines N2i20 in PyR1 and N3i20 in PyR2 (Fig. 2, A and B). These asparagines are predicted to form interdomain H-bonds with polar residues in positions 2i29 and...
129, respectively (Tikhonov et al., 2015). Mutation N3220A in PyR1 decreases sensitivity of the cockroach sodium channel to pyrethroids (Du et al., 2009). Mutation N3220A in PyR2 did not change the channel sensitivity to pyrethroids, but mutation S129A of the putative H-bonding partner of N3220 (Fig. 4), implying that the nitrile group of PyR2-bound DMT is closer to S129 than to N3220.

Conclusions. In this study, we further elaborated the dual-pyrethroid receptor paradigm by creating an atomic model of the mosquito sodium channel with two DMT molecules bound to two different receptors, PyR1 and PyR2, and performing model-driven mutagenesis in PyR1 and PyR2. Our models and experimental data predict a significant degree of rotational symmetry between the two pyrethroid receptor sites, although with subtle differences. In conjunction with findings from previous studies, our results suggest that simultaneous binding of pyrethroids to two receptor sites in the pore module of the insect sodium channel may be necessary to effectively lock the channel in the open state.

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Authorship Contributions
Participated in research design: Zhorov, Dong.
Conducted experiments: Du, Nomura, Zhorov.
Performed data analysis: Du, Nomura, Zhorov.
Wrote or contributed to the writing of the manuscript: Du, Zhorov.

References

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