Functional Expression of an Arachnid Sodium Channel Reveals Residues Responsible for Tetrodotoxin Resistance in Invertebrate Sodium Channels*

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Tetrodotoxin (TTX) is a potent blocker of voltage-gated sodium channels, but not all sodium channels are equally sensitive to inhibition by TTX. The molecular basis of differential TTX sensitivity of mammalian sodium channels has been largely elucidated. In contrast, our knowledge about the sensitivity of invertebrate sodium channels to TTX remains poor, in part because of limited success in functional expression of these channels. In this study, we report the functional characterization in Xenopus oocytes of the first non-insect, invertebrate voltage-gated sodium channel from the varroa mite (Varroa destructor), an ecto-parasite of the honeybee. This arachnid sodium channel activates and inactivates rapidly with half-maximal activation at −18 mV and half-maximal fast inactivation at −29 mV. Interestingly, this arachnid channel showed surprising TTX resistance. TTX blocked this channel with an IC50 of 1 μM. Subsequent site-directed mutagenesis revealed two residues, Thr-1674 and Ser-1967, in the pore-forming region of domains III and IV, respectively, which were responsible for the observed resistance to inhibition by TTX. Furthermore, sequence comparison and additional amino acid substitutions suggested that sequence polymorphisms at these two positions could be a widespread mechanism for modulating TTX sensitivity of sodium channels in diverse invertebrates.

Tetrodotoxin (TTX)2 is a specific and potent blocker of voltage-gated sodium channels, which are essential for the initiation and propagation of action potentials in almost all excitable cells (1). TTX physically occludes the pore of sodium channels and blocks action potential conduction in nerve and muscle (1). The blocking effect of TTX on the sodium channel was first discovered in lobster giant axons (2). However, not all sodium channels are equally sensitive to TTX. For example, among the nine sodium channel isoforms (rNav1.1 to rNav1.9) from rats, TTX has high affinity for rNav1.1–1.4, rNav1.6, and rNav1.7, blocking these channels (TTX-S) at nanomolar concentrations. In contrast, micromolar concentrations of TTX are required to block rNav1.5, rNav1.8, and rNav1.9, the TTX-resistant (TTX-R) sodium channels (3, 4). In this regard, TTX is an effective pharmacological agent that has been used to distinguish different mammalian sodium channel isoforms.

The pore-forming α-subunit of mammalian voltage-gated sodium channels consists of four homologous domains (I–IV), each of which contains six transmembrane segments (S1–S6) and one reentrant P-region connecting S5–S6 (5)(Fig. 1A). In each P-region, the short segments, SS1 and SS2, span the membrane as a hairpin and form the lining of the transmembrane pore (5). Because TTX physically blocks the pore and prevents sodium conductance, elucidation of the TTX receptor site on sodium channels has been invaluable in elucidating the pore structure of sodium channels. In particular, site-directed mutagenesis of the SS1 and SS2 loops of domains I–IV revealed two motifs, DEKA (Asp-384, Glu-942, Lys-1422, and Ala-1714), located in domains I, II, III, and IV, respectively, of rNav1.2) and EEMD (Glu-387, Glu-945, Met-1425, and Asp-1717, located in domains I, II, III, and IV, respectively, of rNav1.2) as major determinants of the TTX receptor site (6, 7) (Fig. 1B). Predominantly negatively charged, the residues in the DEKA and EEMD motifs are equivalently positioned in each of the four domains. In combination, the DEKA and EEMD motifs form inner and outer rings of the sodium channel pore, respectively. The DEKA motif also forms the ion selectivity filter (8). Surprisingly, these two motifs are conserved in both TTX-sensitive and TTX-resistant sodium channels. Instead, a non-aromatic residue, cysteine or serine, immediately downstream of the first Glu in the EEMD motif of rNav1.5, rNav1.8, and rNav1.9 channels is responsible for TTX resistance (9–11).

In contrast to the presence of multiple sodium channel genes in vertebrates, invertebrates, such as jellyfish, flatworms, sea anemones, squid, mites, and insects, generally have fewer sodium channel-encoding genes (12). For example, only one sodium channel gene, para, is found in the fruit fly Drosophila melanogaster (13–16). Nevertheless, recent studies show that insects, such as D. melanogaster and the German cockroach (Blattella germanica), generate functional diversity of sodium channels by alternative splicing and RNA editing of a single sodium channel gene transcript (16–20). Characterization of more than 60 sodium channel variants from both D. melanogaster and B. germanica in Xenopus oocytes shows that all of these variants are highly sensitive to TTX (19, 20). TTX-insensitive sodium currents, however, have been reported in neurons of two jellyfish species (Cyanea capillata and Polyorchis penicillatus), which are the earliest extant organisms to incorporate...
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A nervous system (21–23). In addition, TTX-resistant sodium currents have also been detected in neurons of flatworms (*Bdelloura candida*) (24) and leeches (*Hirudo medicinalis*) (25).

Alignment of the SS2 regions from sequenced invertebrate sodium channels reveals that the outer EEMD motif contains intriguing sequence polymorphisms in domains III and IV, but not in domains I and II (Fig. 1B). For instance, the residue corresponding to Met is a phenylalanine (Phe) in the two jellyfish sodium channels, and a threonine (Thr) in several other invertebrate sodium channels including flatworms and leeches (Fig. 1B). It is not known, however, whether the sequence polymorphisms in the EEMD motif of domains III and IV contribute to TTX insensitivity in invertebrate sodium channels.

To date, insect voltage-gated sodium channels are the only invertebrate sodium channels that have been functionally expressed in an *in vitro* expression system, despite reported attempts (26). Robust functional expression of insect sodium channels in *Xenopus* oocytes requires an accessory subunit, TipE (14). Therefore, it has been generally assumed that an unidentified accessory subunit like TipE may be required for functional expression of non-insect invertebrate sodium channels in *in vitro* (26). Here, we report the functional expression and

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**FIGURE 1. Sequence variations in the P-regions of sodium channels.** A, a diagram of the topology of the α-subunit of voltage-gated sodium channels, which contain four homologous domains (I–IV), each consisting of six transmembrane segments. The short segments SS1 and SS2 in the P-region of each domain are indicated within a circle. The positions of two residues, Ser-1674 and Thr-1967, corresponding to Met-1425 and Asp-1717 in rNav1.2, are indicated. B, amino acid sequence alignment of SS1s and SS2s of all known invertebrate sodium channels. Only part of SS1 is shown. The DEKA and EEMD motifs are shaded. Residues deviating from the EEMD canonical motif are boxed. The *Rattus norvegicus* Na_1.2 sodium channel (GenBank™ accession number, X03639) is included for comparison. Other sequences include: varroa mite *Varroa destructor* (AY259834), fruit fly *D. melanogaster* (M32078), house fly *Musca domestica* (X96668), African malaria mosquito *Anopheles gambiae* (AM422833), yellow fever mosquito *Aedes aegypti* (EU399179), Asian tiger mosquito *Aedes albopictus* (AY663384), domestic silkworm *Bombyx mori* (EU224999), tobacco budworm *Heliothis virescens* (AF017293), diamondback moth *Plutella xylostella* (BAF37093), jewel wasp *Nasonia vitripennis* (NM01134917), red flower beetle *Tribolium castaneum* (XM962937), German cockroach *Blattella germanica* (J35583), southern cattle tick *Rhipicephalus microplus* (AF134216), Manchurian scorpion *Buthus martensi* (AY322171), Chinese bird spider *Ornithoctonus huwena* (DQ839489), California market squid *Loligo opalescens* (L19979), spear squid *Loligo bleekeri* (D14525), TTX-sensitive and -resistant soft shell clam *Mya arenaria* (AAX14719), medicinal leech *H. medicinalis* (AY324424–AY324427), ascidian tunicate *H. roretzi* (D17311), California sea hare *A. californica* (U66915), turbellarian flatworm *B. candida* (U93074), sea anemone *Alaptasia pallida* (AF041851), hydrozoan jellyfish *P. penicillatus* (AF047380), and scyphozoan jellyfish *C. capillata* (L15445).
characterization of an arachnid sodium channel from the varroa mite (Varroa destructor), an ecto-parasite of the honeybee, in Xenopus oocytes. Interestingly, we found that this arachnid sodium channel is highly resistant to TTX. Site-directed mutagenesis enabled us to identify Thr-1674 and Ser-1967 as the molecular basis of TTX resistance of this sodium channel.

**EXPERIMENTAL PROCEDURES**

**Isolation of a Full-length cDNA Clone by Reverse Transcription-PCR**—We previously reported the cloning and sequencing of the coding region of a sodium channel (VdNa,1; formerly called VmNa,1) from the varroa mite (V. destructor) (27). To isolate a full-length cDNA clone for functional expression in Xenopus oocytes, the entire coding region was amplified by reverse transcription-PCR using total RNA isolated from a pool of about 1000 adult mites. The primers used in reverse transcription-PCR were oligo(dT)12–18(RT), tagccggaattcgccgccgctgca (forward primer) and atgtgctctagctagat (reverse primer). The PCR product was cloned into pGH19, a Xenopus oocyte expression vector. A full-length clone was isolated and sequenced in the Research Technology Support Facility at Michigan State University.

**Functional Expression of the VdNa,1 Channel in Xenopus Oocytes**—Because there is no known accessory subunit identified in arachnids that is equivalent to β-subunits in mammals and TipE in insects, we attempted expression of the mite VdNa,1 sodium channel alone by injecting cRNA of VdNa,1 (10 ng/oocyte) into Xenopus oocytes. Sodium currents were recorded by using a standard two-electrode voltage clamp technique. The voltage dependence of activation and fast inactivation were determined using protocols as described previously (28, 29). The data were fitted with a Boltzmann equation to generate V1/2, the midpoint of the activation or inactivation curves, and k, the slope factor. Statistical significance was determined by Student’s t test, and significant values were set at p < 0.05 or as indicated in the table and figure legends.

**RESULTS**

**Functional Expression of the VdNa,1 Channel in Xenopus Oocytes**—Because there is no known accessory subunit identified in arachnids that is equivalent to β-subunits in mammals and TipE in insects, we attempted expression of the mite VdNa,1 sodium channel alone by injecting cRNA of VdNa,1 (10 ng/oocyte) into Xenopus oocytes. Sodium currents were detected 5–7 days after injection, even though the amplitude of peak current was small (less than 1 μA after 7 days of cRNA injection). Nevertheless, the detected sodium currents were sufficient for functional characterization.

The VdNa,1 channel activated and inactivated rapidly; a 20-ms depolarization to −10 mV from the holding potential of −120 mV almost completely inactivated the VdNa,1 channel with a 5–10% non-inactivating current (Fig. 2A). The sodium channel exhibited steep voltage dependence of activation and fast inactivation (Fig. 2B). Interestingly, a significant overlap between the voltage dependence of activation and inactivation was observed (Fig. 2B and Table 1). At −26 mV, the crossing point of the two curves, about 20% of channels were not inacti-
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The VdNa,1 Channel Is TTX Resistant—The concentration of TTX required to completely block the VdNa,1 channel was 50 μM, whereas 10 nM TTX was sufficient to block all current from the BgNa,1-1a and the Drosophila sodium channel, DmNa,1-1 (Fig. 3A). To determine the IC50 of the VdNa,1 channel to TTX, we generated a dose-response curve (Fig. 3A). VdNa,1 channels are ~2,000-fold more resistant to TTX than the two insect sodium channels tested (Fig. 3A, Table 2). No difference was observed between VdNa,1 and VdNa,1a channels in response to TTX (Table 2). Because sodium current expression from VdNa,1 was poor, and deletion of the exon B-like sequence did not affect the TTX activity and channel gating properties, we used VdNa,1a for all subsequent experiments as presented below. We cannot rule out the possibility that deletion of the exon-B-like sequence has an unknown confounding effect with mutations in the pore-forming regions. However, the exon-B-like sequence is located in the second intracellular loop connecting domains II and III. The possibility of TTX sensitivity modulated by deletion of exon-B seems unlikely.

Identification of the Determinants of TTX Resistance of VdNa,1—To elucidate the molecular basis of TTX resistance, we examined amino acid sequences of the P-regions, including TTX-interacting DEKA and EEMD motifs (see Fig. 1). The DEKA motif is conserved in the VdNa,1 channel. However, the EEMD motif is not. Specifically, Met in domain III and Asp in domain IV are substituted with Thr and Ser, respectively. These two substitutions result in an EETS variant motif in the VdNa,1 channel, instead of the canonical EEMD motif in mammalian sodium channels or the EEID variant motif in insect sodium channels (see Fig. 1).

To determine whether these two amino acid substitutions are responsible for TTX resistance of the VdNa,1 channel, we replaced these residues with the corresponding residues in insect or mammalian sodium channels to produce three single mutation channels, T1674I (insect), T1674M (mammal), and S1967D (both insect and mammal), as well as a double mutant channel T1674I/S1967D. All three single substitutions significantly enhanced mutant channel sensitivity to TTX by about 10-fold (Table 2, Fig. 3B). Furthermore, the double mutant channel was about 400-fold more sensitive to TTX than the VdNa,1 channel (Table 2, Fig. 3B). In fact, the level of TTX sensitivity of the double mutant channel is comparable with that of BgNa,1-1a and DmNa,1-1 (Table 2). These results provide strong evidence that substitution of the TTX-binding residues, methionine (isoleucine in insect sodium channels) and aspartic acid, in the mammalian EEMD motif with threonine and serine, respectively, renders the VdNa,1 channel extremely resistant to TTX.

TABLE 2 Sensitivity of wild-type and mutant sodium channels to TTX

| Na+ channel type | IC50 (μM) | n
|-----------------|----------|---
| VdNa,1          | 1.05 ± 0.28 | 5
| VdNa,1a         | 1.45 ± 0.50 | 19
| T1674I          | 0.12 ± 0.03a | 10
| T1674M          | 0.13 ± 0.05a | 7
| T1674F          | 6.95 ± 2.89a | 6
| S1967D          | 0.23 ± 0.08a | 12
| S1967N          | 0.21 ± 0.06a | 11
| S1967A          | 1.58 ± 0.21a | 8
| S1967H          | >100a      | 16
| T1674I + S1967D | 0.00225a   | 10
| BgNa,1-1a       | 0.00055a   | 5
| DmNa,1-1        | 0.00055a   | 5

* n, number of oocytes used.

a Statistically significant difference compared with VdNa,1a. The value represents the mean ± S.D.
alnine residue instead of methionine in domain III and an asparagine residue instead of aspartic acid in domain IV, resulting in an EEFN variant motif. The flatworm BcNa\(_1\) channel also has a threonine in domain III as in VdNa\(_1\), but a histidine residue instead of aspartic acid in domain IV, giving rise to an EETH variant motif. In addition, an alanine instead of aspartic acid in domain IV, giving rise to an EETH variant motif. The flatworm BcNa\(_1\) channel also exhibits a 5–10% non-inactivating current. The flatworm BcNa\(_1\) channel also exhibits a 5–10% non-inactivating current.

Discussion

Prior to this study, insect sodium channels were the only invertebrate sodium channels that had been successfully expressed in Xenopus oocytes for functional characterization. Functional expression of insect sodium channels in Xenopus oocytes relies largely on co-expression of tipE, which encodes a small transmembrane accessory subunit protein (14). It is generally believed that the inability to functionally express other invertebrate sodium channels in Xenopus oocytes is likely because an unknown accessory subunit that is functionally equivalent to TipE is required. Here, we successfully expressed a non-insect invertebrate sodium channel from the varroa mite in Xenopus oocytes. We found that functional expression of the VdNa\(_1\) channel in Xenopus oocytes does not require co-expression of any accessory subunit. Surprisingly, co-expression of the Drosophila TipE with VdNa\(_1\) or VdNa\(_1\)a reduced the amplitude of sodium currents. The possibility that varroa mites express an accessory protein in vivo capable of positively or negatively modulating VdNa\(_1\)a expression cannot be ruled out.

Our analyses showed that VdNa\(_1\) sodium currents exhibited fast activation and inactivation kinetics that are typical of a voltage-gated sodium channel (Fig. 2A). This is not surprising considering that all essential sequences critical for sodium channel function are conserved in VdNa\(_1\), including the ion selectivity motif, the positively charged residues in S4 that form the voltage sensors, and the inactivation particle (27). A particularly striking feature of this channel is the apparent potential window currents have been reported for the rNav1.9 channel in vitro system, such as Xenopus oocytes, for functional characterization. As discussed above, the only functionally characterized invertebrate sodium channels, prior to this study, are insect sodium channels (16, 31). However, no TTX-resistant insect sodium channels have been reported. The marked resistance of the VdNa\(_1\) channel to TTX therefore offers the first opportunity to investigate the molecular basis of TTX resistance in an invertebrate sodium channel. It appears that specific amino acid substitutions in domains III and IV of the EEMD motif represent a major route of evolution toward
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TTX resistance in invertebrate species. In particular, a Thr or Phe substitution of Met in the EEMD motif is present in sodium channels from phylogenetically diverse invertebrates: jellyfish, flatworm, slug, leech, tick, and mite. Substitution of Asp with serine in the EEMD motif is found in sodium channels from the varroa mite and the sea slug (Aplysia californica), and an alanine substitution is found in ascidians (30). Although it remains to be further verified whether these substitutions affect TTX sensitivity of endogenous sodium channels from the respective invertebrate species, our functional assays using recombinant VdNav1a constructs strongly suggest that sodium channels from these invertebrate species possess various degrees of TTX resistance. Accordingly, characterization of molecular determinants of TTX resistance of the VdNav1a channel likely has uncovered a widespread mechanism for modulating TTX sensitivity in invertebrates.

Jellyfish are known to contain TTX-insensitive sodium currents (21–23). Our analysis of VdNa1.1a channels incorporating a methionine to phenylalanine substitution in the EEMD motif, as in jellyfish sodium channels, only reduced, but did not abolish, TTX sensitivity. The methionine to phenylalanine substitution does not entirely account for the TTX insensitivity of jellyfish sodium channels, however, suggesting that other amino acid changes or other mechanisms exist for TTX insensitivity. In mammalian sodium channels, the DEKA motif, which forms the selectivity filter, is also important for modulating TTX sensitivity (5). Neutralization of the Glu residue, charge reversal of Lys, or swapping of Glu and Lys in domains II and III in the DEKA motif renders the rNa1.2 channel extremely resistant to TTX (7, 32). Interestingly, two jellyfish sodium channels possess a DKEA motif, instead of the canonical DEKA motif (see Fig. 1). Therefore, it is possible that a combination of the unique DKEA motif and the methionine to phenylalanine substitution in the EEMD motif results in TTX insensitivity of sodium currents in these species.

Intriguingly, substitution of Met by Thr in the EEMD motif is also found in several vertebrate sodium channels including two pufferfish sodium channels, fuguNav1.4b and tetNav1.4a, and a human skeletal sodium channel, hNav1.7 (Fig. 4). Previous studies predicted that FuguNav1.4b channels would be TTX sensitive because these channels lack the previously identified TTX-resistant residues in domains I or II, which are found in fuguNav1.4a and tetNav1.4b (33). Our results suggest that fuguNav1.4b and tetNav1.4a sodium channels may be TTX resistant as well. The hNav1.7 channel may be TTX resistant, although rat rNa1.7 channels (the counterpart of hNav1.7) contain a canonical EEMD motif and have been shown to be TTX sensitive. In light of our findings, the TTX sensitivity of fuguNav1.4b, tetNav1.4a, and hNav1.7 need to be examined experimentally.

TTX, first isolated from pufferfish species, is found in animals of diverse taxa (34). The widespread detection of TTX-resistant sodium currents and sodium channels in phylogenetically diverse vertebrates and invertebrates suggests the biological and evolutionary importance of TTX resistance. For example, if TTX evolved as a chemical defense against potential predators, we could expect development of TTX resistance in organisms that feed on TTX-bearing food sources. Recent studies of sodium channels in garter snakes, which feed on TTX-bearing newts, illustrate such a scenario (34–36). Several species of newts were found to use TTX to defend against predacious garter snakes. As expected, certain populations of garter snakes that feed on TTX-bearing newts have developed TTX resistance as a result of mutations in the SS2 of domain IV in the outer pore of their skeletal muscle sodium channels (34, 35). Whereas attractive, the universality of this scenario remains to be determined in other species. Our identification and functional confirmation of additional amino acid substitutions in the EEMD motif associated with TTX resistance should facilitate future studies of potential evolutionary prey-predator relationships in diverse natural populations.

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