

# Insect sodium channels and insecticide resistance

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**Abstract** Voltage-gated sodium channels are essential for the generation and propagation of action potentials (i.e., electrical impulses) in excitable cells. Although most of our knowledge about sodium channels is derived from decades of studies of mammalian isoforms, research on insect sodium channels is revealing both common and unique aspects of sodium channel biology. In particular, our understanding of the molecular dynamics and pharmacology of insect sodium channels has advanced greatly in recent years, thanks to successful functional expression of insect sodium channels in *Xenopus* oocytes and intensive efforts to elucidate the molecular basis of insect resistance to insecticides that target sodium channels. In this review, I discuss recent literature on insect sodium channels with emphases on the prominent role of alternative splicing and RNA editing in the generation of functionally diverse sodium channels in insects and the current understanding of the interactions between insect sodium channels and insecticides.

**Keywords** *Para* · *DSCI* · Sodium channel · Knockdown resistance · Alternative splicing · RNA editing

## Introduction

Voltage-gated sodium channels are integral transmembrane proteins responsible for the rapidly rising phase of action potentials, and they are critical for electrical signaling in most excitable cells. In response to membrane depolarization, sodium channels open (activate) and allow sodium ions to flow into the cell, thereby depolarizing the membrane potential. A few milliseconds after channel activation, the channel pore is occluded, a process known as fast inactivation, which is partially responsible for the falling phase of the action potential.

Because of their crucial role in membrane excitability, sodium channels are the target site of a great variety of neurotoxins, such as tetrodotoxin, scorpion toxins, and batrachotoxin, which are produced by plants and animals for defense or predation (Cestele and Catterall 2000; Wang and Wang 2003). Insecticidal pyrethrins, found in extracts of the flowers of *Chrysanthemum* species, also act on sodium channels (Narahashi 1988). Sodium channels are also the primary target of DDT and modern synthetic pyrethroids, which are structural derivatives of the naturally-occurring pyrethrins (Narahashi 1988). Furthermore, recent evidence indicates that a new class of pyrazole-like insecticides, oxadiazines, also target sodium channels (Wing et al. 2005; Silver and Soderlund 2005a).

Due to intensive use of insecticides in arthropod control, many pest populations have developed resistance to these compounds. One major mechanism of resistance is known as knockdown resistance (*kdr*). Insects exhibiting *kdr* have reduced target-site (sodium channel) sensitivity to pyrethroids and DDT resulting

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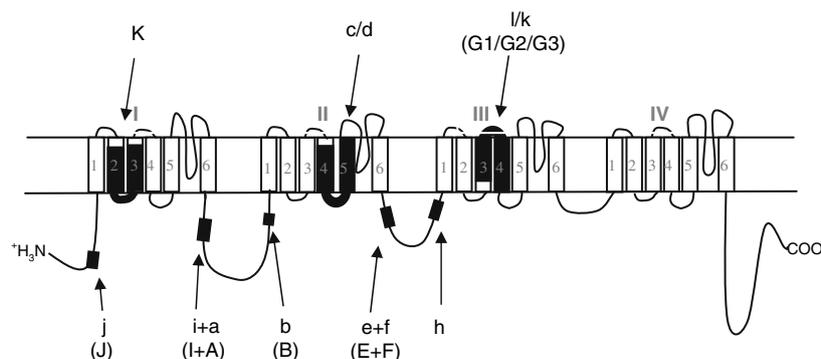
from one or more point mutations in the insect sodium channel protein. The important role of sodium channels in insecticide resistance has prompted many researchers to study insect sodium channel biology. In particular, the past decade has witnessed significant accumulation of knowledge on the functional diversity and pharmacology of insect sodium channels. In this review, I will focus on recent literature that sheds light on the mechanisms of generating functionally diverse sodium channels from a single gene in insects and the current understanding of the interactions between insect sodium channels and insecticides, such as pyrethroids and indoxacarb. Readers are referred to an excellent and complementary review on this topic (Soderlund 2005).

### Structure and function of sodium channels derived from mammalian systems

Extensive molecular analysis of mammalian sodium channels in the last two decades has generated comprehensive views into the structure and function of voltage-gated sodium channels (Catterall 2000; Yu and Catterall 2003; Goldin 2001, 2003). Mammalian sodium channels consist of a large pore-forming transmembrane  $\alpha$ -subunit and several small auxiliary  $\beta$ -subunits. Expression of the  $\alpha$ -subunit alone in *Xenopus* oocytes or mammalian cell lines is sufficient to obtain sodium currents, and the auxiliary  $\beta$ -subunits seem mainly to modulate sodium channel gating and/or protein expression. The  $\alpha$ -subunit contains four homologous domains (named I–IV), each having six membrane spanning segments (named S1–S6) connected by

intracellular or extracellular loops of amino acid sequences (Fig. 1). The selectivity filter and pore are formed by transmembrane segments S5 and S6 together with the membrane-reentrant segments that are part of the loop connecting S5 and S6 of each domain (Fig. 1). Amino acids D, E, K, and A in the loop connecting S5 and S6 of domains I, II, III, and IV, respectively, are the key determinants of ion selectivity. Each S4 segment has five to eight basic residues, either arginine or lysine, separated from each other by two neutral residues. These positively charged S4 segments function as voltage sensors, which move outward in response to membrane depolarization to initiate the opening of the sodium channel. The hydrophobic IFMT motif in the middle of the short linker connecting domains III and IV serves as an inactivation gate and is important for causing fast inactivation by binding to the inactivation gate receptor located within or near the intracellular mouth of the sodium channel pore. Several residues in the intracellular loop connecting S4 and S5 (i.e., IIIS4–S5 and IVS4–S5) are believed to be part of the docking receptor for the inactivation gate.

At least nine different sodium channel  $\alpha$ -subunits ( $\text{Na}_v1.1$  to  $\text{Na}_v1.9$ ) have been identified in mammals (Goldin et al. 2000; Catterall et al. 2003). These sodium channel isoforms exhibit distinct expression patterns in the central and peripheral nervous systems and skeletal and cardiac muscle (Goldin 1999).  $\text{Na}_v1.1$ ,  $\text{Na}_v1.2$ ,  $\text{Na}_v1.3$ , and  $\text{Na}_v1.6$  are predominately expressed in the central nervous system (CNS), whereas  $\text{Na}_v1.7$ ,  $\text{Na}_v1.8$ , and  $\text{Na}_v1.9$  are expressed in the peripheral nervous system (PNS).  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  are expressed in skeletal and cardiac muscles, respec-



**Fig. 1** The schematic diagram of the sodium channel protein indicating four homologous domains (I–IV), each having six transmembrane segments (1–6). The location of segments encoded by the alternative exons are indicated with solid blocks. Optional exons a, b, e, f, h, i, and j, and mutually exclusive exons c/d and l/k identified in *D. melanogaster para* are present in the housefly sodium channel gene *Vssc1* (Lee et al. 2002). Most of

the exons are also found in *BgNa<sub>v</sub>* (Capital letters are used to denote alternative exons in *BgNa<sub>v</sub>*) (Song et al. 2004). However, mutually exclusive exons c/d and optional exon h are not identified in *BgNa<sub>v</sub>*. Corresponding to the l/k site, three mutually exclusive exons G1/G2/G3 are found in *BgNa<sub>v</sub>* (Tan et al. 2002b). Exon K encoding IS2–S3 was found in *BgNa<sub>v</sub>*, but is lacking in *para* and *Vssc1*. See the text for details

tively. Mammalian sodium channel  $\alpha$ -subunit isoforms also exhibit distinct electrophysiological properties (Goldin 2001; Dib-Hajj et al. 2002; Yu and Catterall 2003). Selective expression of different sodium channel genes (with unique gating properties) contributes to the specialized function of sodium channels in various mammalian tissues and cell types (Yu and Catterall 2003).

### Structure and function of insect sodium channels

#### One or two insect sodium channel genes?

In the late 1980s, two putative sodium channel genes, *DSC1* and *para*, were isolated from *Drosophila melanogaster*. *DSC1* was isolated by Larry Salkoff and associates by screening a *D. melanogaster* genomic DNA library using an eel sodium channel gene probe (Salkoff et al. 1987). A *DSC1* orthologous gene was later identified from the German cockroach (*Blattella germanica*) and named *BSC1* (Liu et al. 2001). Like sodium channel  $\alpha$ -subunits, *DSC1* and *BSC1* have four homologous domains, each having six transmembrane segments. Until very recently, *DSC1* and *BSC1* had been predicted to encode a voltage-gated sodium channel based on their overall similarity of deduced amino acid sequence and domain organization to eel and mammalian sodium channel proteins (Littleton and Ganetzky 2000). However, a recent study showed that *BSC1* and *DSC1* encode a novel family of  $\text{Ca}^{2+}$ -selective cation channels, not sodium channels (Zhou et al. 2004; W. Song and K. Dong, unpublished results). All voltage-gated sodium channels, including insect sodium channels, contain amino acids D, E, K, and A in the loops connecting S5 and S6 of domains I, II, III, and IV, respectively, whereas voltage-gated calcium channels contain acidic residues (E, E, E, and E) at the corresponding positions (See Table 2 in Zhou et al. 2004). Site-directed mutagenesis has shown that the E residue in domain III is a critical determinant of the  $\text{Ca}^{2+}$  selectivity of the *BSC1* channel (Zhou et al. 2004). Interestingly, several other putative sodium channels in invertebrates, including *ApCSN1* (*Aiptasia pallida*, sea anemone), *CYNA1* (*Cyanea capillata*), *PpSCN1* (*Polyorchis penicillatus*, Hydrozoan jellyfish), and *LbNa<sub>v</sub>1* (*Loligo bleekeri*, squid), all possess an E in domain III (Refs. in Zhou et al. 2004). Therefore, it seems likely that the other invertebrate *BSC1/DSC1*-like channels may also exhibit  $\text{Ca}^{2+}$  selectivity, although functional expression of these channels remains to be established. The biological role of *DSC1* and *BSC1* channels in insects also remains to be deter-

mined. However, a recent study showed that partially reduced expression of the *DSC1* gene was correlated with certain olfactory defects in *D. melanogaster* (Kulkarni et al. 2002), suggesting a role of *DSC1* in olfaction.

The *para* gene was isolated in Barry Ganetzky's laboratory from mutants exhibiting a temperature-sensitive paralytic phenotype (Loughney et al. 1989). The overall structure of and the amino acid sequence of the *Para* sodium channel shares a high similarity with those of mammalian sodium channel  $\alpha$ -subunits. The structural features that are critical for mammalian sodium channel function, including residues crucial for sodium selectivity, are conserved in the *Para* sodium channel (Loughney et al. 1989). Most importantly, subsequent functional expression and characterization in *Xenopus* oocytes conclusively demonstrated that *para* encodes a sodium channel (Feng et al. 1995; Warmke et al. 1997). Except for significant sequence similarities with the *DSC1* channel protein and limited sequence similarity with putative  $\text{Ca}^{2+}$  channel  $\alpha 1$  subunits, *Para* is not similar to any other genes in the *Drosophila* genome (Littleton and Ganetzky 2000). Therefore, it seems that *para* is the only gene that encodes the sodium channel in *Drosophila* and presumably in other insect species as well.

Because of the intimate involvement of sodium channels in insecticide resistance (see below), *para*-orthologous genes have been isolated from several medically or agricultural important insect pest species (Soderlund and Knipple 2003). However, in most cases, only partial cDNA clones were obtained. Full-length cDNA clones are available only for three *para* orthologues: *Vssc1* from the house fly (Ingles et al. 1996), *BgNa<sub>v</sub>* from the German cockroach (Dong 1997); and *VmNa<sub>v</sub>* from the varroa mite (Wang et al. 2003). The availability of the full-length clones made it possible to successfully express these *para* orthologues in *Xenopus* oocytes and to demonstrate that these genes indeed encode functional sodium channels (Ingles et al. 1996; Tan et al. 2002a; Song et al. 2004; Du et al., unpublished).

#### TipE: an auxiliary subunit?

Robust expression of insect sodium channels in *Xenopus* oocytes requires a small transmembrane protein, TipE, which has two transmembrane segments connected by a large extracellular loop and intracellular amino and carboxyl termini (Feng et al. 1995). Coexpression of TipE with *Para* in oocytes increases the amplitude of peak current and modifies the kinetics of fast inactivation (Feng et al. 1995; Warmke et al.

1997), suggesting that TipE functions as an auxiliary subunit like the  $\beta$ -subunits of mammalian sodium channels. A TipE ortholog cloned from the house fly appears to fulfill the same role as TipE (Lee et al. 2000). TipE also enhances the expression of BgNa<sub>v</sub> in oocytes (Tan et al. 2002a). These results suggest the functional conservation of these transmembrane proteins in different insect species.

Recently, four TipE homologs (TEH1, 2, 3, 4) are found in *D. melanogaster* and their predicted secondary structures resemble those of TipE (Derst et al. 2006). Like TipE, TEH1, 2, and 3, but not TEH4, increase the expression of a Para variant in oocytes (Derst et al. 2006). TEH1 also alters voltage-dependent inactivation and recovery from inactivation of the Para variant. Interestingly, except for TEH1, which is only expressed in the central nervous system, TEH2, 3, 4 are widely expressed in both neuronal and non-neuronal tissues. The involvement of these proteins in regulating sodium or other ion currents in insect tissues/cells remains to be investigated.

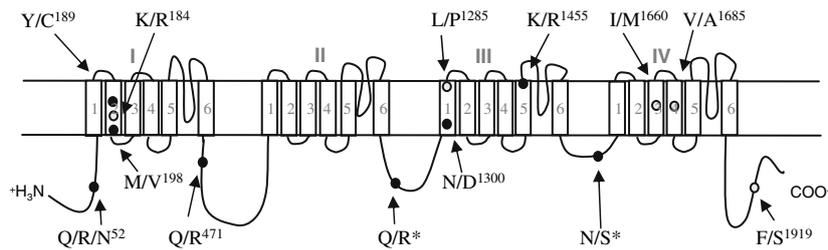
### Extensive alternative splicing of insect sodium channel gene transcripts

Alternative splicing of the *para* transcript was first reported in the *D. melanogaster* (Thackeray and Ganetzky 1994). To date, a total of nine alternatively splice sites—a, b, c/d, i, j, e, f, h, l/k—have been identified in *para* (Fig. 1; Thackeray and Ganetzky 1994, 1995; O'Dowd et al. 1995; Lee et al. 2002). Exons, a, b, i, j, e, f, and h, are optional and are located in the intracellular linkers, whereas the two mutually exclusive exons c/d and l/k are situated in the transmembrane regions. Interestingly, most of these alternative splice sites are conserved in *D. virilis* (Thackeray and Ganetzky 1995), the house fly *Vssc1* gene (Lee et al. 2002), and the cockroach *BgNa<sub>v</sub>* gene (Tan et al. 2002a; Song et al. 2004). Such a high degree of conservation, however, does not seem to extend to beyond insect sodium channels. For example, only two of the splice sites, corresponding to b and l/k in *Drosophila*, are found in the *VmNa<sub>v</sub>* (varroa mite sodium channel) transcript, which has three unique splice sites (Wang et al. 2003; Y. Du et al., unpublished results). Although the splice sites are highly conserved in insect species, the frequency of exon usage can be quite different. For example, more than 60% of the *para* (both *D. melanogaster* and *D. virilis*) and *Vssc1* transcripts contain exon b, but less than 20% of BgNa<sub>v</sub> transcripts contain exon B (Capital letters are used to denote alternative exons in BgNa<sub>v</sub>).

At the position corresponding to the *Drosophila* l/k splice site, which is located in IIS3–S4, there are three mutually exclusive exons, called G1, G2, and G3, in *BgNav* (in Fig. 1; Tan et al. 2002b). Exons G1 and G2 share 65% amino acid sequence homology, and correspond to exons l and k in *Drosophila*, respectively. In contrast, exon G3 has no sequence homology with either G1 or G2, and possesses a stop codon that would generate a truncated protein containing only the first two domains. Interestingly, at this conserved splicing site, VmNa<sub>v</sub> contains an optional alternative exon (named exon 3) that encodes IIS3–S4 (Wang et al. 2003). Because IIS4 is essential for sodium channel function, exclusion of exon 3 likely also generates a non-functional channel. Furthermore, the exon/intron arrangement of this region in insects bears a striking similarity with that of the genomic region coding for IIS3–S4 of the vertebrate sodium channel protein, Na<sub>v</sub>1.6 (Scn8a), of mice, humans, and fish (Plummer et al. 1997). Like exon G3 in *BgNav*, the mammalian counterparts possess a stop codon that would generate a truncated protein containing only the first two domains. Therefore, alternative splicing in this region appears to be of a very ancient origin and is preserved in organisms ranging from mites and insects to humans.

### RNA editing of insect sodium channel transcripts

RNA editing is a well-known post-transcriptional modification that could significantly change protein function by introducing site-specific alterations in gene transcripts. RNA editing results in the conversion of one base to another, or the insertion and deletion of nucleotides (Bass 2001). Both A-to-I and U-to-C editing events have been reported for insect sodium channel genes. Ten A-to-I editing sites are identified in the *para* transcript in *D. melanogaster* by direct sequencing or restriction enzyme digestion analysis of partial cDNA clones (Palladino et al. 2000; Hanrahan et al. 2000; Reenan et al. 2000). The actual number of RNA editing sites may be much greater than ten, because the region examined in these studies represents only a portion of the complete *para* open reading frame. Eight of the ten editing events result in amino acid changes in the transmembrane segments or intracellular linkers (Fig. 2). Some of these RNA editing sites are conserved in *D. virilis* (Hanrahan et al. 2000); however, possible functional alterations caused by these amino acid changes remain to be examined. Surprisingly, none of these A-to-I editing sites are found in BgNa<sub>v</sub>. Instead, two unique A-to-I RNA editing sites and three U-to-C editing sites are identified in BgNa<sub>v</sub>, each resulting in amino acid changes in



**Fig. 2** RNA editing sites in Para and BgNa<sub>v</sub>. Ten A-to-I editing sites have been identified in para, eight of which result in amino acid changes (*solid dots*). The amino acid positions of these editing sites are not numbered in the original publications (Palladino et al. 2000; Hanrahan et al. 2000). Here, six sites are numbered according to the amino acid sequence of the GenBank sequence (accession number: M32078). \*We cannot give amino

acid positions for R/K and N/S sites because there are more than one R or N residues in these regions. Therefore, only the approximate locations are indicated. Two A-to-I editing sites (K/R<sup>148</sup> and I/M<sup>1660</sup>) and three U-to-C editing sites (L/P<sup>1285</sup>, V/A<sup>1685</sup>, and F/S<sup>1919</sup>) are identified in BgNa<sub>v</sub>. F/S<sup>1919</sup> is also identified in para (Liu et al. 2004). See the text for details

the transmembrane segments (Fig. 2; Song et al. 2004; Liu et al. 2004). One of the U-to-C editing sites, F/S<sup>1919</sup>, is also found in *para* (Liu et al. 2004). It is not clear whether similar RNA editing events occur in mammalian sodium channel transcripts.

#### Functional consequences of alternative splicing and RNA editing of insect sodium channels

As discussed above, selective expression of different sodium channel genes (with unique gating properties) contributes to the functional diversity of sodium channels in various mammalian tissues and cell types (Yu and Catterall 2003). Similarly, electrophysiological recordings in different insect neurons also revealed distinct sodium currents, indicating heterogeneity in sodium channel properties in insects (Byerly and Leung 1988; Saito and Wu 1991, 1993; Schafer et al. 1994; O'Dowd et al. 1995; Le Corrionc et al. 1999; Lapied et al. 1990, 1999; Wicher et al. 2001; Grolleau and Lapied 2000; Defaix and Lapied 2005). It is still unclear how insects produce distinct sodium currents from a single sodium channel gene. One possibility is that alternative splicing and RNA editing generate sodium channels with unique gating properties. Recent functional characterization in *Xenopus* oocytes of several cockroach sodium channel variants that undergo splicing and/or RNA-editing suggests that this is indeed the case. For example, electrophysiological characterization of 20 BgNa<sub>v</sub> splice variants in *Xenopus* oocytes revealed an impressive spectrum of differences in the level of sodium current expression and gating properties (e.g., the voltage-dependence of activation and/or inactivation) (Song et al. 2004). One variant, BgNa<sub>v</sub>11, activates at more negative membrane potentials (e.g., -60 mV), whereas others require more membrane depolarization (e.g., -45 mV) for channel activation. Similarly, the half-maximal volt-

ages for steady-state inactivation range from -37 to -60 mV. BgNa<sub>v</sub>7 has a significant overlap between the voltage dependences of activation and steady-state inactivation, with  $V_{1/2}$  values of -36.7 and -37.2 mV, respectively. Consequently, this variant produces a large window current over a range of -45 to -25 mV, which could lead to membrane depolarization at sub-threshold potentials associated with oscillatory activities, summation of synaptic input, or modulation of firing frequency (Crill 1996).

The role of specific alternative exons in modulating sodium channel gating has been demonstrated. Exon B in BgNa<sub>v</sub> encodes an eight-amino acid sequence in the linker connecting domains I and II. BgNa<sub>v</sub> variants containing exon-B expressed poorly in oocytes (Song et al. 2004), but deletion of exon-B caused the level of sodium current expression to increase dramatically (Song et al. 2004). Similarly, introduction of exon-B into variants lacking exon-B resulted in the reduction of peak sodium currents of these recombinant channels (Song et al. 2004). These results indicate exon-B plays a critical role in the regulation the level of sodium channel expression and/or the size of current in *Xenopus* oocytes. Considering that the location and sequence of this exon is highly conserved in *Drosophila para* and also in house fly *Vssc1* (Lee et al. 2002; Song et al. 2004), the role of exon b/B in regulating insect sodium current expression may be universal. Exon b/B, located in the first intracellular linker, contains a consensus sequence for phosphorylation. Down-regulation of sodium current expression by protein kinases has been documented for mammalian sodium channels (Numann et al. 1991; West et al. 1991; Smith and Goldin 1996, 1997, 2000). Exon b/B could serve as a regulatory on-or-off switch to control the neuronal excitability by responding to second messengers to meet unique physiological needs in specific tissues or cell types. In this regard, it is interesting to note that

the majority of optional exons in insect sodium channels are located in intracellular linkers. These linker sequences seem ideal for interaction with other cellular proteins, such as G-proteins and kinases, and for integrating cell and tissue-specific signals to “customize” sodium channel properties.

The functional consequence of RNA editing of insect sodium channel transcripts has also been shown recently. A U-to-C RNA editing event at the F/S site at the C-terminal domain of BgNa<sub>v</sub> generated a non-inactivating (persistent) Na<sup>+</sup> current (Liu et al. 2004). Eliminating the U-to-C editing at the L/P site in IIS3 caused the BgNa<sub>v</sub>1-1 variant to activate and inactivate at more hyperpolarizing membrane potentials. Similarly, abolishment of the A-to-I editing at the K/R site in IS2 shifted the voltage-dependence of activation of the BgNa<sub>v</sub>1-2 variant to a more hyperpolarized potential. Clearly, these editing events are responsible for the different activation and inactivation properties of the BgNa<sub>v</sub>1-1 and BgNa<sub>v</sub>1-2 variants. The modulation of sodium channel gating properties by specific RNA editing events supports the hypothesis that RNA editing is involved in the fine-tuning of the neuronal activity (Seeburg 2000).

In short, recent studies suggest that the functional plasticity of the insect sodium channel is generated primarily by alternative splicing and RNA editing of a single gene transcript. Whereas mammals rely on multiple sodium channel genes to produce functionally distinct isoforms, insects seem to depend on extensive alternative splicing and RNA editing of a single sodium channel gene to presumably fulfill customized sodium channel activities in different cell types and tissues.

### Sodium channels as insecticide targets

Sodium channels are targets of a variety of neurotoxins including naturally occurring toxins from animals and plants, therapeutic drugs, and synthetic insecticides pyrethroids and oxadiazines. These neurotoxins are grouped based on their distinct binding sites on the sodium channel and/or unique effects on sodium channel function. The extensive body of work on this topic is discussed in several excellent reviews (Gordon 1997; Zlotkin 1999; Cestele and Catterall 2000; Wang and Wang 2003).

#### Pyrethroids

Pyrethroids are synthetic structural derivatives of insecticidal pyrethrins present in the pyrethrum extract of *Chrysanthemum* species (Elliott 1977). They are

grouped into two categories (type I and type II) based on their distinct poisoning symptoms, effects on nerve preparations, and their chemical structures (Gammon et al. 1981; Lawrence and Casida 1982; for review see Narahashi 1986). Type I pyrethroids lack an  $\alpha$ -cyano group which is present at the phenylbenzyl alcohol position of type II pyrethroids. Type I pyrethroids cause repetitive discharges in response to a single stimulus, while type II pyrethroids caused a membrane depolarization accompanied by a suppression of the action potential. Historically, studies on the mode of action of pyrethroids have been conducted using vertebrate and non-insect invertebrate nerve preparations. Collectively, these studies show that pyrethroids cause prolonged opening of sodium channels primarily by inhibiting channel deactivation and stabilizing the open configuration of the sodium channel (for review see Narahashi 1986, 1988, 1996, 2000; Soderlund and Bloomquist 1989; Raymond-Delpech et al. 2005). The prolonged channel opening is evidenced by a large tail current associated with repolarization under voltage-clamp conditions. Furthermore, voltage-clamp experiments showed that type II pyrethroids inhibit the deactivation of sodium channels to a greater extent than type I pyrethroids. The decay of tail currents induced by type II pyrethroids is at least one order of magnitude slower than those induced by type I pyrethroids. These quantitative differences in tail-current decay kinetics between type I and type II pyrethroids may account for their different actions on the nervous system. However, the molecular basis of the differential effects of type I and type II pyrethroids on sodium channels remains to be determined.

Recent studies on the mechanism of action of pyrethroids on insect sodium channels expressed in oocytes and the molecular mechanism of *kdr* (see below) confirmed that sodium channels are the target of pyrethroid insecticides. Vais et al. (2000a) showed that deltamethrin, a potent type II pyrethroid, preferably bind to the activated (open) state and inhibit deactivation of Para sodium channels expressed in oocytes. Similar effects were also observed on house fly and cockroach sodium channels (Smith et al. 1997; Tan et al. 2002a, b; Tan et al. 2005). Furthermore, these studies also demonstrated that the effects of type I and type II pyrethroids on insect sodium channels are similar to those observed from electrophysiological studies using non-insect nerve preparations.

#### Oxadiazines

Commercialization of indoxacarb, a new insecticide, represents a success in the development of a new

generation of sodium-channel-targeted insecticides (McCann et al. 2001). In insects, indoxacarb is metabolically converted to *N*-decarbomethoxylated JW062 (DCJW), a more active metabolite. Mammals convert indoxacarb into nontoxic metabolites, which contributes to its selective toxicity to insect pests.

Recent studies showed that the sodium channel is a major cellular target of indoxacarb and DCJW (Wing et al. 2005; Silver and Soderlund 2005a). For example, indoxacarb reduces the compound action potential in the *M. sexta* larval nerve cord preparation and inhibits firing of abdominal stretch receptor organs in larvae of the fall armyworm *Spodoptera frugiperda*, but the effect was slower than that of its metabolite, DCJW (Wing et al. 1998, 2005). Both indoxacarb and DCJW blocked sodium channels in patch-clamped neurons isolated from rat dorsal root ganglia (Nagata et al. 1998; Tsurubuchi and Kono 2003; Zhao et al. 2003) and from the central nervous system (CNS) of the American cockroach neurons (Lapied et al. 2001; Zhao et al. 2005). These studies suggest that the action of indoxacarb and DCJW share similarities with those of RH3421, a dihydropyrazole insecticide (Salgado 1992), and local anesthetics (LA), which block sodium currents in a state-dependent manner, by preferably binding to the inactivated state of the sodium channel (Hille 1977). Studies using the rat Na<sub>v</sub>1.4 and cockroach sodium channel variants expressed in *Xenopus* oocytes confirmed the state-dependent block of both mammalian and insect sodium channels by DCJW (Silver and Soderlund 2005b; Song et al. 2006; Silver and Soderlund 2006).

### Pyrethroid sensitivities among naturally occurring sodium channel variants

Differential sensitivities of mammalian sodium channel isoforms to pyrethroid insecticides are well known. For example, tetrodotoxin (TTX)-sensitive sodium channels in the rat dorsal root ganglion neurons are less sensitive to pyrethroids than TTX-resistant sodium channels in the same neurons (Ginsburg and Narahashi 1993; Tatebayashi and Narahashi 1994; Song and Narahashi 1996). Rat Na<sub>v</sub>1.2 and Na<sub>v</sub>1.4 channels are insensitive to pyrethroids (Warmke et al. 1997; Vais et al. 1997; Smith and Soderlund 1998; Wang et al. 2001), whereas rat Na<sub>v</sub>1.8 sodium channels are more sensitive to pyrethroids (Choi and Soderlund 2006). Interestingly, substitution of an isoleucine in IIS4–S5 in the rat Na<sub>v</sub>1.2 channel by a methionine (which is in insect sodium channels) increased channel sensitivity to pyrethroids (Vais et al. 2000b). However, the iso-

leucine residue is conserved in all known mammalian sodium channel protein, and is therefore cannot be responsible for the differential sensitivities of mammalian sodium channel isoforms. Clearly, an important future goal would be to determine other amino acid residues involved in modulating the pyrethroid sensitivity of various mammalian sodium channel isoforms.

The existence of sodium channel variants in insects that exhibit distinct levels of sensitivity to pyrethroids has been demonstrated recently. In cockroaches, the BgNa<sub>v</sub>2-1 channel variant is 100-fold less sensitive to deltamethrin than the BgNa<sub>v</sub>1-1 variant (Tan et al. 2002b). As described above, the BgNa<sub>v</sub>2-1 channel contains a mutually exclusive exon G2, whereas the BgNa<sub>v</sub>1-1 channel contains exon G1. The differential pyrethroid sensitivities between BgNa<sub>v</sub>1-1 and BgNa<sub>v</sub>2-1 channels can be partially attributed to the presence of exon G1 or G2 (Tan et al. 2002b). More recently, a V1356A change in exon G2 is identified to be responsible for the exon G2-associated low sensitivity of the BgNa<sub>v</sub>2-1 channel to pyrethroids (Du et al. 2006). Because there are ten scattered exon-G-independent amino acid differences between BgNa<sub>v</sub>1-1 and BgNa<sub>v</sub>2-1, systematic site-directed mutagenesis are necessary to identify additional residues that are involved in the different sensitivity between the two variants. The BgNa<sub>v</sub>2-1 variant appears to be a minor variant in cockroaches (Song et al. 2004; Z. Liu and K. Dong, unpublished result) and is expressed mainly in gut and ovary (Tan et al. 2002b). The insensitivity to pyrethroids of BgNa<sub>v</sub>2-1 is likely caused by unique channel gating properties of this variant, which are presumably customized for specific roles in gut and ovary. Regardless of their precise roles in mammalian and insect neurophysiology, pyrethroid-resistant sodium channel isoforms or variants are proving to be a unique resource for the elucidation of the interaction between sodium channels and pyrethroids at the molecular level.

### Pyrethroid resistance caused by naturally selected *kdr* mutations in the sodium channel

*kdr* mutations confer reduced neuronal sensitivity to pyrethroids and DDT in insects (Soderlund and Bloomquist 1990). In the early 1990s, several groups conducted genetic linkage analysis and showed that *kdr* mutations were linked to the *para*-orthologous genes in several insect species (Refs. in Soderlund and Knipple 2003). Subsequent molecular analyses led to the identification of multiple point mutations in the *para*-orthologous genes that are associated with *kdr*

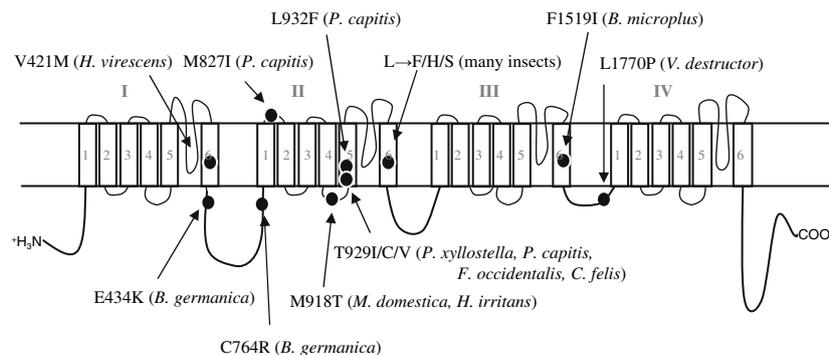
and *kdr*-type resistance to pyrethroids in these insects (Soderlund 2005). To experimentally confirm the involvement of *kdr*-associated mutations in pyrethroid resistance, a number of laboratories used the *Xenopus* oocyte expression system to examine the sensitivity of mutant sodium channels to pyrethroids. To this end, ten sodium channel mutations have been confirmed to be responsible for *kdr* and *kdr*-type resistance (Fig. 3).

The most common *kdr* mutation is a leucine (L) to phenylalanine (F), histidine (H), or serine (S) substitution in domain II segment 6 (IIS6), associated with low levels of pyrethroid resistance. The L to F mutation is present in *kdr* house flies, *Musca domestica* (Williamson et al. 1996; Miyazaki et al. 1996); German cockroaches, *Blattella germanica* (Miyazaki et al. 1996; Dong 1997; Dong et al. 1998); diamondback moths, *Plutella xylostella* (Martinez-Torres et al. 1997, 1999); peach-potato aphids, *Myzus persicae* (Schuler et al. 1998; Martinez-Torres et al. 1999a); mosquitoes, *Anopheles gambiae* (Martinez-Torres et al. 1998; Ranson et al. 2000), *Cluex pipiens* (Martinez-Torres et al. 1999b) and *Culex quinquefasciatus* (Xu et al. 2005); horn flies, *Haematobia irritans* (Guerrero et al. 1997); Colorado potato beetles, *Leptinotarsa decemlineata* (Lee et al. 1999a); western flower thrips, *Frankliniella occidentalis* (Forcioli et al. 2002); codling moth, *Cydia pomonella* (Brun-Barale et al. 2005); and cat fleas, *Ctenocephalides felis* (Bass et al. 2004). The L to S mutation is found in *C. pipiens* (Martinez-Torres et al. 1999b) and *A. gambiae* (Ranson et al. 2000). The L to H mutation is present in tobacco budworms, *Heliothis virescens* (Park and Taylor 1997). In *Xenopus* oocytes, the L to F/H mutation in IIS6 reduces the pyrethroid sensitivity of Para, Vssc1 and BgNa<sub>v</sub> sodium

channels by five- to ten-fold (Smith et al. 1997; Zhao et al. 2000; Vais et al. 2000a; Tan et al. 2002a; Liu et al. 2002).

In highly resistant strains, additional sodium channel mutations are often found to co-exist with the L to F mutation in IIS6. For example, in *super-kdr* house fly strains, which exhibit greater levels of resistance to DDT and pyrethroids than *kdr* houseflies, a methionine (M) to threonine (T) mutation (M918T) in the linker connecting IIS4 and IIS5 is present in addition to the L to F mutation (L1014F) in IIS6 (Williamson et al. 1996; Miyazaki et al. 1996). The same M to T mutation together with the L to F mutation is also found in a horn fly strain that exhibited high-level resistance to pyrethroids (Guerrero et al. 1997). In *Xenopus* oocytes, the double mutations drastically reduced the sensitivity of the house fly and *Drosophila* sodium channels to pyrethroids (Lee et al. 1999c; Vais et al. 2000a). Interestingly, the M918T mutation alone also reduces sodium channel sensitivity to pyrethroids to some extent (Lee et al. 1999c; Vais et al. 2001), but it has not been found alone in any population of pyrethroid-resistant insects.

In the German cockroach, additional resistance mutations have been discovered that reduce the sensitivity of sodium channels in conjunction with the L to F mutation mentioned above. Four point mutations, aspartic acid to glycine (D58G) at the N-terminus, glutamic acid to lysine (E434K) and cysteine to arginine (C764R) in the linker between IS6 and IIS1, and proline to leucine (P1888L) at the C-terminus, together with the L to F mutation (L932F) in IIS6, are associated with high levels of resistance (Liu et al. 2000). Unlike the housefly M918T mutation, E434K and



**Fig. 3** *kdr* mutations in insect sodium channels. Only those *kdr* mutations that have been confirmed to reduce the sodium channel sensitivity to pyrethroids are indicated (solid dots). Positions of M827I, M918T, T929I, and L932F mutations are numbered according to the amino acid sequence of the housefly Vssc1 sodium channel protein (Ingles et al. 1996; Williamson et al. 1996). Positions of E434K, C764K, and F1519I mutation

are numbered according to the amino acid sequence of the cockroach sodium channel protein (Dong 1997). V421M is numbered according to the amino acid sequence of the tobacco budworm sodium channel protein (Park et al. 1997). L1770P is numbered according to the amino acid sequence of the varroa mite sodium channel protein (Wang et al. 2003). See the text for details

C764R by themselves do not reduce the sensitivity of the cockroach sodium channel to pyrethroid in *Xenopus* oocytes. However, when either the E434K or C764R mutation is combined with the L993F mutation (i.e., KF or RF), the channel sensitivity is reduced by 100-fold (Tan et al. 2002a), and concomitant presence of all three mutations (KRF) reduces channel sensitivity to pyrethroid by 500-fold (Tan et al. 2002a). Whether the two mutations (D58G and P1888L) at the N- and C- terminus contribute to pyrethroid resistance remains to be determined.

In a highly resistant *P. xylostella* strain, a threonine to isoleucine (T929I) mutation in IIS5 is found along with the L to F mutation in IIS6 (Schuler et al. 1998). The T929I mutation alone drastically reduces the Para sodium channel to pyrethroids and is considered a *super-kdr* mutation (Vais et al. 2001). At the same position, a T to C change and a T to V change is detected in western flower thrips (Forcioli et al. 2002) and a highly pyrethroid-resistant cat flea population (Bass et al. 2004), respectively. The same T to I mutation is also found in pyrethroid-resistant head lice, *Pediculus capitis* along with two more mutations, M827I and L932F (Lee et al. 2000, 2003; Tomita et al. 2003; Yoon 2006). The M827I + T929I + L932F triple mutant Vssc1 channel is completely insensitive to pyrethroids (Yoon 2006).

It is important to note that some *kdr*-type strains lack the L to F/H/S mutation in IIS6. Instead, a valine (V) to M change at 421 in IS6 is found in several pyrethroid-resistant *H. virescens* populations (Park et al. 1997). The V421M mutation reduces pyrethroid sensitivity by ten-fold when introduced into *Drosophila*, housefly and cockroach sodium channels (Zhao et al. 2000; Lee and Soderlund 2001; Liu et al. 2002). The L to F/H/S mutation is also not found in any pyrethroid-resistant arachnids. Instead, an F to I mutation in IIS6 is present in several pyrethroid-resistant southern cattle tick (*Boophilus microplus*) strains (He et al. 1999). This mutation completely abolishes the pyrethroid sensitivity of cockroach sodium channels to structurally diverse pyrethroids (Tan et al. 2005). Finally, four amino acid changes in the sodium channel have been identified in pyrethroid resistant varroa mites, an ectoparasite of honey bees, including an L to P mutation in the third linker connecting domains III and IV (Wang et al. 2002). Interestingly, pyrethroid-susceptible insects already possess a P at the corresponding position. This is intriguing as varroa mites are more sensitive to the pyrethroid fluralinate (which is used in the control of mites) than honey bees (Santiago et al. 2000). Indeed, substitution of P with L makes the cockroach sodium channel more

sensitive to pyrethroids (Liu et al. 2006). These results indicate that the L/P residues are at least partially responsible for the differential sensitivities of insects and mites to pyrethroids.

In summary, both common and unique mutations in sodium channel genes are found to be responsible for pyrethroid resistance in different insect and arachnid pest species. The information is valuable for monitoring the frequency of pyrethroid resistance in field pest populations as part of pest resistance management. New *kdr* mutations will likely be identified in other agricultural and medically important arthropod pests as pyrethroids continue to be used as a major pest control strategy.

### Do *kdr* mutations define the pyrethroid-binding site(s)?

Studies are being conducted to understand how the *kdr* mutations alter sodium channel sensitivity to pyrethroids. These mutations could confer sodium channel resistance to pyrethroids by reducing the binding of pyrethroids to the sodium channel and/or by counteracting the action of pyrethroids via a binding-independent mechanism (e.g., by altering sodium channel gating). Although a high-affinity pyrethroid-binding site was detected on the  $\alpha$ -subunit of rat brain sodium channel preparations ten years ago (Trainer et al. 1997), direct measurement of pyrethroid binding affinity and capacity to insect sodium channels has not been achieved, because the high lipophilicity of pyrethroids results in extremely high levels of nonspecific binding to membranes and filters and consequently masks any specific pyrethroid binding (Pauron et al. 1989; Rossignol 1988; Dong 1993). As an alternative strategy, electrophysiological analyses of *kdr* mutant channels in oocytes are employed to quantify the effects of *kdr* mutations on the channel gating and pyrethroid binding. Vais et al. (2000a) showed that *kdr* (L1014F) in IIS6 and *super-kdr* mutations M918T in the IIS4–S5 linker and T929I in IIS5 all enhanced closed-state inactivation, thereby reduced channel opening, which is required for the action of pyrethroids. These mutations also reduced the binding affinity for open channels (Vais et al. 2000a, 2003). Utilizing the competitive binding of active 1R-cis and inactive 1S-cis permethrin isomers to the sodium channel, Tan et al. (2005) showed that L993F in IIS6 and F1519W in IIS6 reduced pyrethroid binding to the cockroach sodium channel. The findings from these studies suggest that these particular residues are part of the pyrethroid binding site. However, it is possible that

some of these residues do not physically interact with pyrethroids, but they are critical for the sodium channel conformation required for the binding of pyrethroids. A recent study used the crystal structures of potassium channels to generate a housefly sodium channel model in the open conformation (O'Reilly et al. 2006). This model highlights the role of IIS4–S5 linker and the IIS5 and IIS6 helices in pyrethroid binding, supporting the involvement of M918, T929 and F1519 in pyrethroid binding. The model also predicts that several additional residues in IIS5 and IIS6 could be part of the pyrethroid binding site and might contribute to the different sensitivities between insect and mammalian sodium channels. Further experiments are needed to verify these predictions.

## Conclusion

Since the seminal cloning of the *Drosophila para* gene 18 years ago, excellent progress has been made in understanding the biology of insect sodium channels. Successful functional expression in *Xenopus* oocytes and the identification of TipE as an auxiliary protein have had a major impact on many aspects of research on insect sodium channels. A recent significant development is the demonstration that alternative splicing and RNA editing are two main mechanisms by which insects generate a remarkable array of structurally and functionally diverse sodium channel variants from a single sodium channel gene. Determining how alternative splicing and RNA editing of the sodium channel gene shapes the electrical activity of specific neurons involved in specific sensory, motor, and integrative functions in vivo is an exciting next step for future research. Also, after two decades of speculation, the functional identity of the DSC1 channel has been resolved. The discovery that DSC1 is a novel calcium-selective cation channel now begs for research aimed at determining its precise function in insect development, physiology and behavior.

Without doubt, the great interest in insect sodium channels in the past decade is in part prompted by an exciting connection between pyrethroid resistance and sodium channel mutations. The intensive research efforts in this area have greatly improved our understanding of the molecular basis of pyrethroid resistance. An impressive collection of *kdr* or *kdr*-type sodium channel mutations have been identified and confirmed to be involved in reducing sodium channel sensitivity to pyrethroids. The naturally occurring pyrethroid-resistant sodium channel variants provide a

complementary approach for understanding the interaction of pyrethroids with insect sodium channels at the molecular level. Collectively, these insect sodium channel mutations and variants provide an excellent foundation for further studying the interaction of sodium channels with pyrethroids. It seems certain that the next few years will witness continuing progress on the elucidation of the structural features in sodium channels that are required for the binding/action of pyrethroids.

Sodium channels are excellent target sites for a variety of neurotoxins, in addition to pyrethroids. There are at least nine known toxin binding sites on the sodium channel, which could be potential target sites of new insecticides. Indoxacarb/DCJW is an example of a new chemical which successfully exploits a binding site on the sodium channel unique from other classes of insecticides. Additionally, several scorpion toxins are selectively active on insect sodium channels, but not on their mammalian counterparts (Bosmans et al. 2005; Strugastsky et al. 2005). The unique pharmacology displayed by insect sodium channels can serve to provide leads for new insecticide discovery and development. Furthermore, study of the action of novel neurotoxins in turn should enhance our basic understanding of sodium channel gating and pharmacological properties unique to insects.

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