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Mutations in the transmembrane helix S6 of domain IV confer cockroach sodium channel resistance to sodium channel blocker insecticides and local anesthetics

Dingxin Jiang[1,*], **Yuzhe Du**[2,*], **Yoshiko Nomura**[2], **Xingliang Wang**[3], **Yidong Wu**[3], **Boris S. Zhorov**[4], and **Ke Dong**[2]

[1]Key Laboratory of Natural Pesticide and Chemical Biology, Ministry of Education, Laboratory of Insect Toxicology, South China Agricultural University, Guangzhou, China

[2]Department of Entomology, Neuroscience and Genetics Programs, Michigan State University, East Lansing, Michigan, USA

[3]College of Plant Protection, Nanjing Agricultural University, Nanjing, China

[4]Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, Ontario, Canada; and Sechenov Institute of Evolutionary Physiology & Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia

Abstract

Indoxacarb and metaflumizone are two sodium channel blocker insecticides (SCBIs). They preferably bind to and trap sodium channels in the slow-inactivated non-conducting state, a mode of action similar to that of local anesthetics (LAs). Recently, two sodium channel mutations, F1845Y ($F^{4i15}Y$) and V1848I ($V^{4i18}I$), in the transmembrane segment 6 of domain IV (IVS6), were identified to be associated with indoxacarb resistance in *Plutella xylostella*. F^{4i15} is known to be critical for the action of LAs on mammalian sodium channels. Previously, mutation $F^{4i15}A$ in a cockroach sodium channel, $BgNa_v1$-1a, has been shown to reduce the action of lidocaine, a LA, but not the action of SCBIs. In this study, we introduced mutations $F^{4i15}Y$ and $V^{4i18}A/I$ individually into the cockroach sodium channel, $BgNa_v1$-1a, and conducted functional analysis of the three mutants in *Xenopus* oocytes. We found that both the $F^{4i15}Y$ and $V^{4i18}I$ mutations reduced the inhibition of sodium current by indoxacarb, DCJW (an active metabolite of indoxacarb) and metaflumizone. $F^{4i15}Y$ and $V^{4i18}I$ mutations also reduced the use-dependent block of sodium current by lidocaine. In contrast, substitution $V^{4i18}A$ enhanced the action metaflumizone and lidocaine. These results show that both $F^{4i15}Y$ and $V^{4i18}I$ mutations may contribute to target-site resistance to SCBIs, and provide the first molecular evidence for common amino acid determinants on insect sodium channels involved in action of SCBIs and LA.

Abstract

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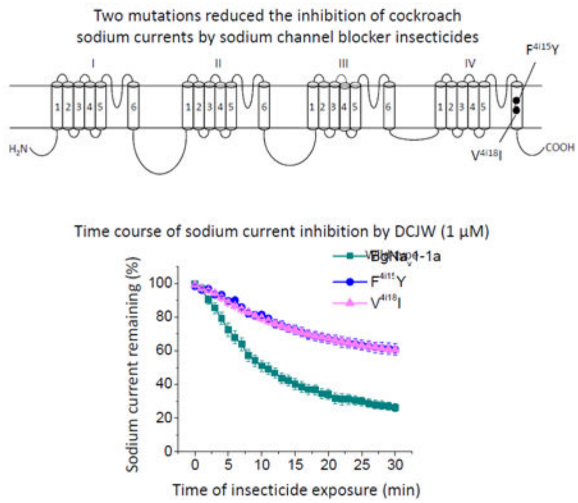
*The authors contributed equally to the work



Introduction

Voltage-gated sodium channels are critical for the initiation and propagation of action potentials in nerves and other excitable cells. Like mammalian sodium channels, insect sodium channels are comprised by four homologous domains (I-IV), each having six membrane spanning helical segments (S1-S6) (Catterall, 2014; Dong et al., 2014). In response to membrane depolarization, the S4 segments move outward, initiating the opening of the activation gate, which is formed by cytoplasmic ends of each S6 (i.e., activation). Within a few milliseconds, sodium channels close or inactivate, which is known as fast inactivation. Prolonged depolarization, however, causes sodium channels enter into a different inactivated state, slow inactivation, that is distinct from fast inactivation. Recovery from fast inactivation takes tens of milliseconds, whereas recovery from slow inactivation requires seconds to minutes of membrane repolarization to return to a resting state (Goldin, 2003; Vilin and Ruben, 2001).

Indoxacarb and metaflumizone are two sodium channel blocker insecticides (SCBIs; Fig. 1). Indoxacarb, the first registered insecticide of this class, causes cessation of feeding, poor coordination, paralysis, and death (Harder et al., 1996; Narahashi, 2001; Silver and Soderlund, 2005; Wing et al., 2005) in a wide range of agricultural pests. Indoxacarb, a proinsecticide, is activated within insects to its more potent, N-decarbomethoxylated metabolite, DCJW (Fig. 1) (Wing et al., 2005; Wing et al., 2000; Wing et al., 1998). Metaflumizone, the second commercialized SCBI, causes poisoning symptoms that are similar to those produced by indoxacarb (Salgado and Hayashi, 2007). Interestingly, SCBIs share a similar mode of action with local anesthetics (LAs), such as lidocaine, anticonvulsants and antiarrhythmics (Salgado, 1992; Salgado and Hayashi, 2007; Wing et al., 2005; Wing et al., 2000; Wing et al., 1998). LAs and related drugs interrupt the initiation and propagation of nerve impulses (i.e., action potentials) by blocking sodium channels, thereby relieving or preventing pain (Catterall, 1987). These compounds preferentially block open and inactivated states of the sodium channel and have a lower affinity to channels in the resting state (Fozzard et al., 2005; Hille, 2001). Similarly, SCBIs inhibit sodium channel function by binding selectively to slow-inactivated states (Silver et al., 2010).

LAs, anticonvulsants and antiarrhythmics bind to a receptor in the inner pore of sodium channels and impede ion permeation (Catterall, 2012). Site-directed mutagenesis studies with mammalian sodium channels revealed that the receptor site for these compounds is formed by amino acid residues in the S6 segments in domains I, III and IV (Catterall, 2012; Mike and Lukacs, 2010). In particular, two LA-sensing residues in IVS6, i.e., F1764 and Y1771 in rat $Na_v1.2$ and F1579 and Y1586 in $Na_v1.4$, are critical for the binding and action of LAs and related drugs on mammalian sodium channels. To facilitate recognition of these mutations among sodium channels from various species, here we use a nomenclature universal for P-loop ion channels (Du et al., 2013; Zhorov and Tikhonov, 2004). It provides a common designation of the two residues in various sodium channels as F^{4i15} and Y^{4i21}, where 4i denotes the domain 4 inner helix (IVS6), and 15 and 21 are the relative numbers of the residues in IVS6.

Soderlund and associates have investigated the role of F^{4i15} and Y^{4i21} in the action of SCBIs on mammalian sodium channels (Silver and Soderlund, 2007; von Stein et al., 2013). Similar to the effect on the action of LAs, alanine substitution, $F^{4i15}A$, resulted in a significant reduction in the ability of DCJW and RH3421, a different experimental SCBI, to inhibit $Na_v1.4$ sodium channels expressed in *Xenopus* oocytes (Silver and Soderlund, 2007). In contrast, mutation of the tyrosine residue, Y^{4i21}, to alanine in $Na_v1.4$ channels resulted in a significant increase in the potency of indoxacarb, DCJW, and RH3421 (Silver and Soderlund, 2007). Mutational analysis of F^{4i15} and Y^{4i21} in a cockroach sodium channel, $BgNa_v1$-1a, revealed that neither $F^{4i15}A$ or $Y^{4i21}A$ reduce the action of SCBIs on $BgNa_v1$-1a channels (Silver et al., 2009). Nevertheless, both $F^{4i15}A$ and $Y^{4i21}A$ reduce the use-dependent block by lidocaine of $BgNa_v1$-1a channels (Song et al., 2011). These results suggest that these two residues contribute to the LA receptor site in insect sodium channels, but have a limited role in the action of SCBIs.

Recently, we identified two sodium channel mutations, $F^{4i15}Y$ and $V^{4i18}I$ (Fig. 2), which were associated with high levels of resistance to SCBIs in field populations of the diamondback moth (*P. xylostella*) in China (Wang et al., 2015). Particularly, one population of *P. xylostella* (BY12) collected from Baiyun, Guangdong province of China in 2012, was 750-fold more resistant to indoxacarb and 70-fold more resistant to metaflumizone compared with a susceptible strain (Wang et al., 2015). Both mutations, F1845Y and V1848I in IVS6 (i.e., $F^{4i15}Y$ and $V^{4i18}I$), were detected in the BY12 population. Furthermore, a significant correlation between allele frequencies of the two mutations and levels of resistance to both indoxacarb and metaflumizone was observed in multiple field-collected populations (Wang et al., 2015). Interestingly, F^{4i15} corresponds to the major LA-sensing residue in mammalian and cockroach sodium channels (Fig. 2). Valine V^{4i18} is three positions downstream of F^{4i15}. The $F^{4i15}A$ substitution did not confer $BgNa_v1$-1a channels resistance to SCBIs (Silver et al., 2009). However, it remains unknown whether $F^{4i15}Y$ and/or $V^{4i18}I$ mutations alter the action of SCBIs on sodium channels.

Functional expression of sodium channels from the diamondback moth has not been established yet. Therefore, in this study we introduced the $F^{4i15}Y$ and $V^{4i18}A/I$ mutations into a well-characterized cockroach sodium channel, $BgNa_v1$-1a, and conducted functional analysis of the mutant channels in *Xenopus* oocytes using two-electrode voltage clamp. Both naturally occurring mutations, $F^{4i15}Y$ and $V^{4i18}I$, introduced individually were found to reduce the ability of indoxacarb, DCJW and metaflumizone to inhibit sodium current. In contrast, the $V^{4i18}A$ mutation did not alter the action of indoxacarb and DCJW, but enhanced the inhibitory effect by metaflumizone. In addition, mutations $F^{4i15}Y$ and $V^{4i18}I$ were found to reduce the use-dependent block of sodium current by lidocaine, whereas the $V^{4i18}A$ mutation enhanced the blocking affect by lidocaine. These results demonstrate that F^{4i15} and V^{4i18} are involved in the action of both SCBIs and lidocaine, suggesting that SCBIs and lidocaine share overlapping receptor sites on the sodium channel.

Materials and Methods

Site-directed mutagenesis

Site-directed mutagenesis was performed by PCR using specific primers and Phusion High-Fidelity DNA polymerase (NEB, Ipswich, MA). All mutants were verified by DNA sequencing.

Expression of $BgNa_v$ Sodium Channels in *Xenopus laevis* Oocytes

The procedures for oocyte preparation, cRNA synthesis and injection are identical to those described previously (Tan et al., 2002). For robust expression of the $BgNa_v$ sodium channel, cRNA was co-injected into oocytes with *Drosophila melanogaster* tipE cRNA (1:1 ratio), which enhances the expression of insect sodium channels in oocytes (Feng et al., 1995; Warmke et al., 1997).

Electrophysiological Recording and Analysis

Sodium currents were recorded using the two-electrode voltage clamp technique. Electrodes were pulled from borosilicate glass and filled with 3 M KCl and 0.5% agarose. Resistances ranged between 0.5 and 1.5 MΩ. Currents were measured with an oocyte clamp amplifier OC725C (Warner Instrument Corp., Hamden, CT), Digidata 1440A (Axon Instruments, Foster City, CA), and pClamp 10.2 software (Axon Instruments). Capacitive transient and leak currents were subtracted using the P/N (N = 4) subtraction method.

Examination of $BgNa_v$ channel sensitivity to SCBIs and lidocaine

The methods for measuring the effects of SCBIs and lidocaine on $BgNa_v1$-1a channels are similar to those described in Silver et al. (2009) and Song et al. (2011), respectively. Briefly, we measured the onset of block by SCBIs at or near the potential of 50% steady-state inactivation. After establishing a stable voltage clamp near the half-inactivation potential specific to a channel variant, insecticide-containing solution was perfused into the bath at a rate of 3 ml/min over the first 7-8 min whereas the time course of onset of block was recorded for 30 min.

For use- and frequency dependence of block, we measured peak current by delivering a train of 50 pulses (a 20 ms test pulse to −10 mV from the holding potential of −120 mV) at a frequency of 20 Hz or at a range of frequencies between 1 and 20 Hz, respectively. The amplitude of sodium current elicited by each pulse was then normalized to the amplitude of the peak sodium current generated by the initial pulse.

All experiments were performed at room temperature. Indoxacarb and DCJW were provided by K. D. Wing and D. Cordova (DuPont Agrochemicals) and metaflumizone was provided by V. Salgado (BASF Agricultural Products). Lidocaine was purchased from Sigma (L-7757). Drugs and insecticides were perfused onto oocytes in a manner similar to that previously described (Tatebayashi and Narahashi, 1994).

Data are presented as mean ± SEM. Statistical analysis was determined using a one-way ANOVA test and Scheffe's post hoc analysis. Significance values were set at $p < 0.05$ or as indicated in the table and figure legends.

Molecular modeling

A homology model of the cockroach sodium channel variant $BgNa_v1$-1 was constructed based on the crystal structure of the Na_vAb sodium channel (PMID: 22678295) as described elsewhere (Du et al., 2011) .

Results

3.1. F^{4i15}Y and V^{4i18}I mutant channels are more resistant to indoxacarb, DCJW and metaflumizone than wild type channels

We introduced F^{4i15}Y and V^{4i18}A/I into a cockroach sodium channel, $BgNa_v1$-1a, and first examined the effects of the mutations on the gating properties. All three mutant channels generated sodium currents in *Xenopus* oocytes that were sufficient for functional analysis. Compared to the wild-type, none of the mutations altered the voltage dependence of activation or fast or slow inactivation (Figs. 3; Table 1). However, both F^{4i15}Y and V^{4i18}I mutations caused incomplete slow inactivation. As shown in Figure 3B, about 30% of sodium currents remained at -30 mV and beyond for F^{4i15}Y channels and the modification in slow activation was less extensive for V^{4i18}I channels.

Figure 4A shows a representative trace of $BgNa_v1$-1a sodium current elicited by a 20 ms depolarization to -10 mV from a holding potential of -120 mV. Perfusion of oocytes with 1 μM DCJW for 30 min at a holding potential of -120 mV, did not alter the amplitude of $BgNa_v1$-1a currents (Fig. 4A), indicating DCJW had no effect on sodium channels in the resting state. The same results were observed from the three mutants (data not shown). However, perfusion with 1 μM of DCJW for 30 min at -55 mV (the $V_{1/2}$ of slow inactivation for $BgNa_v1$-1a channels) or -60 mV (the $V_{1/2}$ of slow inactivation for mutant $BgNa_v1$-1a channels) caused a gradual decrease in $BgNa_v1$-1a current (Fig. 4B). This is consistent with findings from previous studies (Silver et al., 2009), confirming that SCBIs inhibit $BgNa_v1$-1a channels by binding to inactivated states. We then compared the state-dependent inhibition of sodium channels by indoxacarb, DCJW, or metaflumizone between wild-type,

$F^{4i15}Y$ and $V^{4i18}I/A$ channels at depolarized holding potentials during 30 min of insecticide exposure.

Figure 4C shows the time courses of inhibition of $BgNa_v1$-1a wild-type and $F^{4i15}Y$ and $V^{4i18}A/I$ mutant channels by 1.0 μM DCJW. For both $BgNa_v1$-1a and $V^{4i18}A$ channels, inhibition of sodium current increased steadily after DCJW exposure. No inhibition was observed in the absence of DCJW (data not shown). After 30 min exposure of WT and $V^{4i18}A$ channels to 1.0 μM DCJW, peak sodium currents decreased by ~74% and 70%, respectively, whereas peak sodium current in the $F^{4i15}Y$ and $V^{4i18}I$ channels decreased by only 39 - 40% (Fig. 4C and Table 2). However, 10 μM DCJW reduced the currents in the $F^{4i15}Y$ and $V^{4i18}I$ mutants by about 77% and 74%, respectively (Fig. 4D and Table 2). This effect was comparable with inhibition of sodium currents by 1 μM DCJW on the WT channels (Table 2), indicating that the mutants were ~10- fold less sensitive to DCJW than the WT channel. Furthermore, unlike the naturally occurring mutations, $F^{4i15}Y$ and $V^{4i18}I$, the $V^{4i18}A$ mutation did not alter the inhibition by DCJW (Fig. 4C).

Apparently, the extent of inhibition by indoxacarb and metaflumizone on the wild-type and $F^{4i15}Y$ and $V^{4i18}I$ mutant channels are similar. Following 30 min of exposure to 10 μM indoxacarb, $BgNa_v1$-1a sodium currents were reduced by about 64% (Fig. 4E), whereas $F^{4i15}Y$ and $V^{4i18}I$ currents were reduced only by about 23% and 37%, respectively (Fig. 4E and 4G). 10 μM metaflumizone inhibited peak sodium currents in the WT, $F^{4i15}Y$ and $V^{4i18}I$ channels by ~ 57, 24 and 26%, respectively (Fig. 4F, G and Table 2). Unlike the $V^{4i18}I$ mutant channels, the degree of inhibition of $V^{4i18}A$ channels by indoxacarb was similar to that of $BgNa_v1$-1a channels (Fig, 4E and 4G). In contrast, 10 μM metaflumizone inhibited the $V^{4i18}A$ channels by 75%, which is more than that in WT channels (Fig. 4F and 4G).

3.2. $F^{4i15}Y$ and $V^{4i18}I$ mutant channels were resistant to lidocaine, but $V^{4i18}A$ channels were more sensitive to lidocaine

It is well established that LAs preferentially bind to open and inactivated states of the mammalian sodium channel and cause their use-dependent and frequency-dependent block (Li et al., 1999). Here we employed rapid trains of stimulus pulses to promote binding of lidocaine to open and inactivated channels and compared the responses of $F^{4i15}Y$ and $V^{4i18}I/A$ mutant channels with those of $BgNa_v1$-1a. As shown in Fig. 5, inhibition of $BgNa_v1$-1a channels by lidocaine gradually enhanced with the increase of pulse numbers or frequency. However, little enhancement was observed for the $F^{4i15}Y$ and $V^{4i18}I$ channels with either changes in pulse number or frequency. In contrast, inhibition of $V^{4i18}A$ mutant channels by lidocaine was stronger than that for the WT channel at all pulse numbers or frequencies (Fig. 5).

3.3. Possible location of binding sites for SCBIs

Figure 6 shows side (Fig. 6A) and top (Fig. 6B) views of the Na_vAb-based homology model of the $BgNa_v1$-1 channel with space-filled sidechains of F^{4i15} and V^{4i18}. For comparison, the space-filled models of DCJW are shown in two projections, in the same scale as the channel model. The V^{4i18} sidechain faces the inner pore, whereas the F^{4i15} sidechain may move between the inner pore and the III/IV domain interface. Location of F^{4i15} and V^{4i18}

suggests that SCBIs bind in the inner pore and may expand a hydrophobic moiety into the III/IV domain interface.

Discussion

The mode of action of SCBIs is different from those of other classes of insecticides that act on sodium channels, including pyrethroid insecticides. Therefore, SCBIs have been excellent alternatives for controlling insect pest populations which have developed resistance to pyrethroid insecticides due to target-site modifications (Wing et al., 2005). However, in recent years, resistance to SCBIs began to emerge in field populations of various lepidopteran pests, including *Plutella xylostella* (Khakame et al., 2013; Santos et al., 2011; Sayyed and Wright, 2006; Zhao et al., 2006), *Spodoptera litura* (Shad et al., 2012; Tong et al., 2013) and *Spodoptera exigua* (Che et al., 2013; Tong et al., 2013; Zhou et al., 2011). More recently, two sodium channel mutations, F1845Y and V1848 (i.e., $F^{4i15}Y$ and $V^{4i18}I$), were found to be associated with SCBI resistance in diamondback moth populations in China (Wang et al., 2015). This study represents the first effort to characterize the effect of naturally occurring sodium channel mutations on the action of SCBIs. Our functional analysis of the mutations in cockroach sodium channels expressed in *Xenopus* oocytes show that both $F^{4i15}Y$ and $V^{4i18}I$ mutations reduced the potency of indoxacarb, DCJW and metaflumizone, indicating that these mutations likely contribute to SCBI resistance in diamondback moth populations. The findings from Wang et al. (2015) and this study provide the molecular evidence for target-site modification as a major mechanism of SCBI resistance, and the two mutations could be used as molecular markers for resistance monitoring in field populations of the diamondback moth and possibly in other pest species.

In insects, indoxacarb is metabolically converted to the more active metabolite DCJW (Wing et al., 2005). While DCJW is a more potent blocker of sodium channels, it is well-documented in the literature that indoxacarb exhibits a modest level of blocking effect on most mammalian sodium channel isoforms expressed in *Xenopus* oocytes (von Stein et al., 2013) and in mammalian neurons (Zhao et al., 2003). Indoxacarb also inhibits cockroach sodium channels in primary neurons (Zhao et al., 2005) and cockroach sodium channels expressed in *Xenopus* oocytes (Silver et al., 2009). The effects of indoxacarb on wild-type cockroach sodium channels from our current study are consistent with those reported in our previous study (Silver et al., 2009).

Identification of these naturally occurring mutations in sodium channels are also valuable resources for elucidating the molecular basis of binding and action of SCBIs on sodium channels. Systematic site-directed mutagenesis of residues using alanine substitutions have been successfully employed in identification of major residues for LA binding in mammalian sodium channels. A number of residues in the S6 transmembrane segments of domains I, III, and IV are thought to affect therapeutic drug activity directly in rat $Na_V1.2$ sodium channels (Ragsdale et al., 1994, 1996; Yarov-Yarovoy et al., 2001; Yarov-Yarovoy et al., 2002), and similar results have been reported for other mammalian sodium channel isoforms, including the $Na_V1.4$ sodium channel (Nau et al., 1999; Wang et al., 2004; Wang et al., 2000; Wang and Wang, 1998). Because of similar modes of action between LA and SCBIs, residues necessary for LA activity have been used as a road map to determine their potential roles in the binding and action of SCBIs on insect sodium channels. However, alanine substitution of the key LA-sensing residue, F^{4i15}, did not reduce the $BgNa_v1$-1a channel sensitivity to SCBIs (Silver et al., 2009), but reduced the effect of lidocaine (Song et al., 2011). In fact, the F^{4i15}A substitution caused a slight (1.3-fold) increase in $BgNa_v1$-1a sodium channel sensitivity to DCJW. These earlier findings demand further evaluation on the role of F^{4i15} in the action of SCBIs on insect sodium channels (Silver et al., 2009). Here by examining a different substitution, F^{4i15}Y, which emerged naturally due to intensive use of indoxacarb in controlling agricultural insect pests, we demonstrated that F^{4i15} is involved in the action of SCBIs on insect sodium channels.

Another significant finding of this study is that both F^{4i15}Y and V^{4i18}I mutations also confer $BgNa_v$ channels resistance to a local anesthetic, providing further molecular evidence for overlapping receptors for SCBI and LA on sodium channels (Salgado and Hayashi, 2007; von Stein et al., 2013; Wing et al., 2005). Different substitutions at V^{4i18} affect the sensitivity of $BgNa_v1$-1a channels to different SCBIs and lidocaine differently. The V^{4i18}I substitution confers resistance to lidocaine and all three SCBIs, whereas V^{4i18}A substitution enhanced the sensitivity of $BgNa_v1$-1a channels to metaflumizone and lidocaine, but had little effect on the sensitivity of $BgNa_v1$-1a channels to indoxacarb or DCJW, indicating that changes at this amino acid affect both SCBI and LA activity and imply that both SCBIs and LA occupy this space in voltage-sensitive sodium channels.

Our mutational and electrophysiological analyses suggest that the SCBIs may interact with valine V^{4i18} and phenylalanine F^{4i15}. Same-scale images of the Na_vAb-based model of the insect sodium channel pore module (Du et al., 2013) and DCJW suggest that the ligand may directly interact with the pore-facing F^{4i15} and V^{4i18} (Fig. 6). The finding that mutation F^{4i15}A has little effect on the action of indoxacarb and DCJW, but enhances the action of metaflumizone (Silver et al., 2009) suggests that repulsing and attractive forces may be balanced for interactions of F^{4i15} with indoxacarb and DCJW, while metaflumizone repulsion from F^{4i15} may overbear attraction to F^{4i15}. This proposition would be consistent with the large size of SCBI molecules that could tightly fit into the inner pore. Mutation F^{4i15}Y may destabilize SCBIs due to unfavorable interactions of their hydrophobic moieties with the hydrophilic hydroxyl of tyrosine. The fact that mutation V^{4i18}I decreases the potency of SCBIs also suggests that large SCBIs fit tightly into the inner pore. A ligand may form a close contact with V^{4i18}, but repel from the bigger I^{4i18}. Mutation V^{4i18}A increases the potency of metaflumizone indicating that the ligand experiences repulsion from the bulky V^{4i18}, but not from the small A^{4i18}. Further research is needed to test these hypotheses.

The effects of $BgNa_v1$-1 mutations on the use- and frequency-dependent block by lidocaine (Fig. 5) are consistent with the open-state model of $Na_v1.4$ in which the ligand binds in the inner pore between IS6, IIIS6 and IVS6, whereas its diethylamine, amide and dimethylphenyl moieties approach the pore-facing residues F^{4i15}, V^{4i18} and Y^{4i22}, respectively (Tikhonov and Zhorov, 2007). Lidocaine, which is much smaller than SCBIs (Fig. 1), is unlikely to experience van der Waals repulsions from inner pore residues. Mutation F^{4i15}Y would impose unfavorable contact between the tyrosine hydrophilic group and hydrophobic groups of lidocaine. Similarly, the V^{4i18}I mutation may shift the ligand farther from IVS6, thus deteriorating its interactions with F^{4i15} and Y^{4i22}, whereas mutation V^{4i18}A would allow a closer contacts of the ligand with IVS6 and stronger interactions with F^{4i15} and Y^{4i22}.

In conclusion, here we functionally established that the potency of SCBIs on $BgNa_v$1-1a channels was reduced by two naturally occurring sodium channel mutations that are associated with SCBI resistance in the diamondback moth. Our study also provides important information on ligand-channel interactions, suggesting that the two mutations likely alter the binding of SCBIs to its receptor site on voltage-sensitive sodium channels. Our results also provide molecular evidence for the notion that the receptor sites of SCBIs and LAs overlap on insect sodium channels. Further modeling and mutational analysis are needed to define the receptor sites of these compounds on insect sodium channels.

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- Mutations $F^{4i15}Y$ and $V^{4i18}I$ reduced the sensitivity of cockroach sodium channels to SCBIs.
- The two mutations also confer cockroach sodium channel resistance to lidocaine.
- SCBIs and lidocaine share a common receptor site on cockroach sodium channels.

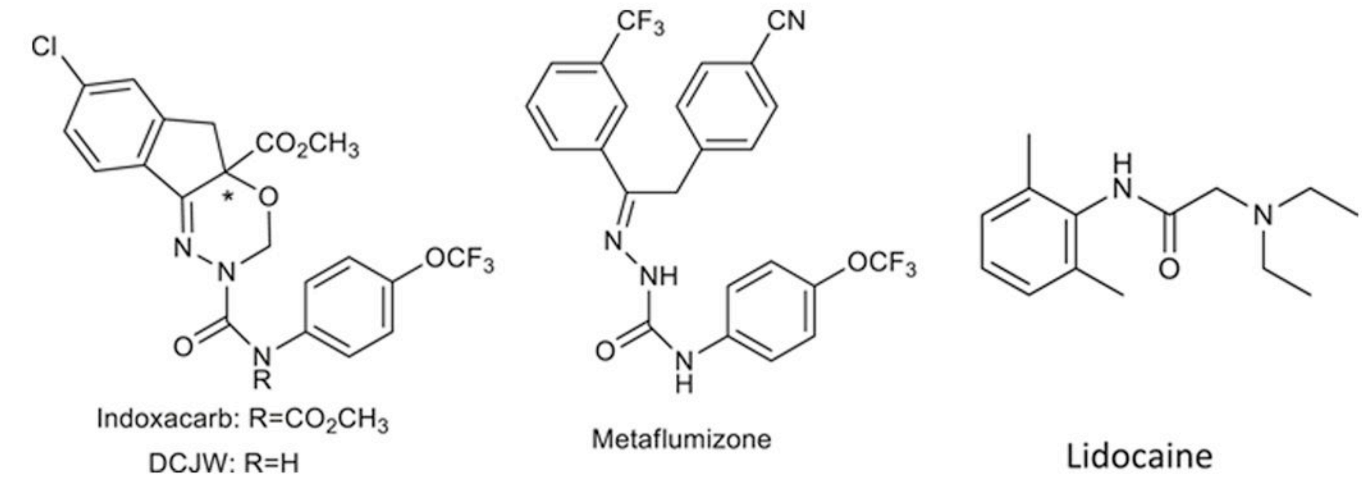

Figure 1.

Chemical structures of indoxacarb, DCJW, metaflumizone and lidocaine.

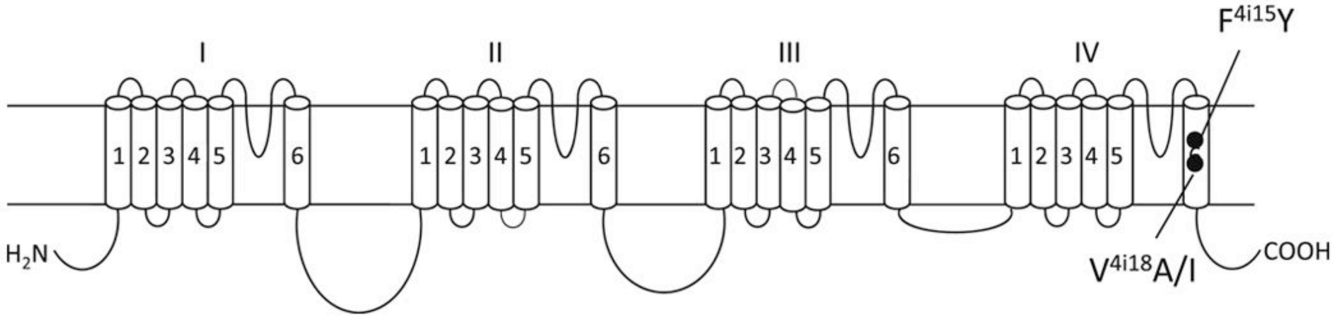

Figure 2.

The topology of BgNa$_V$1-1a indicating the positions of two naturally occurring mutations, F^{4i15}Y and V^{4i18}I, which are associated with resistance to indoxacarb in *P. xylostella* (Wang et al., 2015). F^{4i15}Y and V^{4i18}I are labeled based on the nomenclature universal for P-loop ion channels (Du et al., 2013; Zhorov and Tikhonov, 2004). A residue label includes the domain number (1– 4), segment type (k, the linker-helix between S4 and S5; i, the inner helix S6; and o, the outer helix S5), and relative number of the residue in the segment.

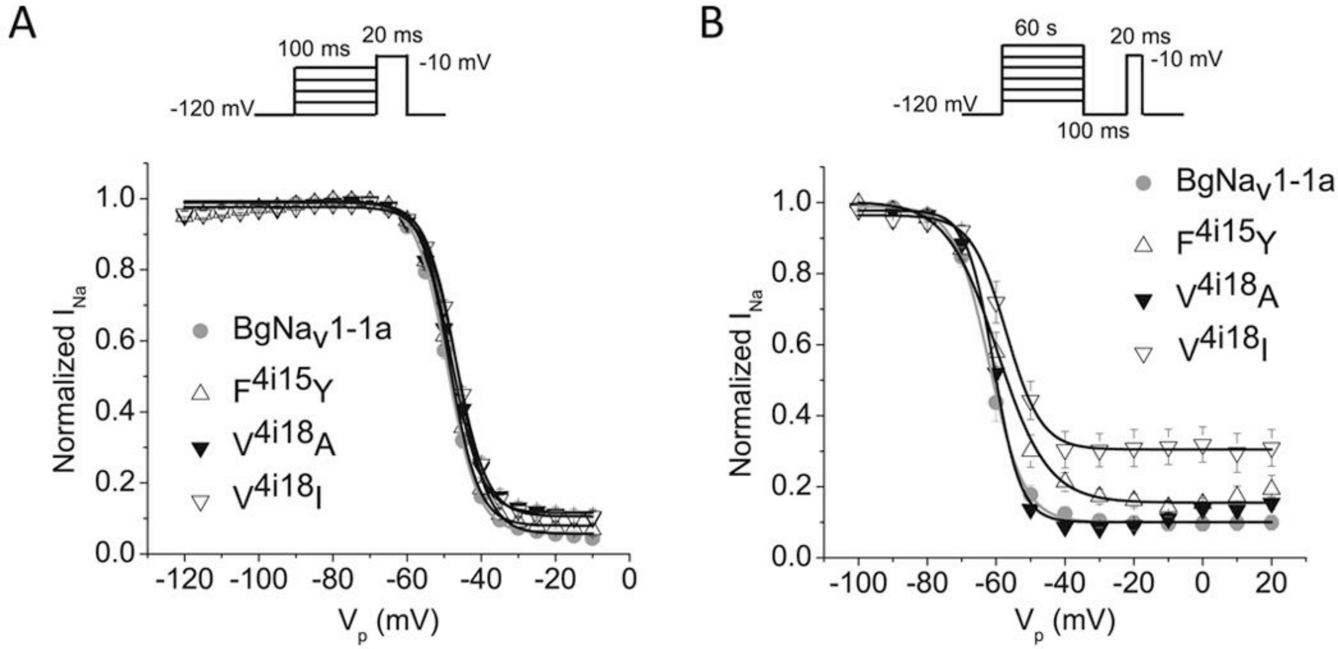

Figure 3.

Effect of $F^{4i15}Y$ and $V^{4i18}A/I$ substitutions on the voltage dependence of fast (A) and slow (B) inactivation of $BgNa_v1$-1a channels. **A.** Voltage dependence of fast inactivation. **B.** Voltage dependence of slow inactivation. The voltage dependences were measured using a series of prepulse potentials (Vp) as indicated in the recording protocols.

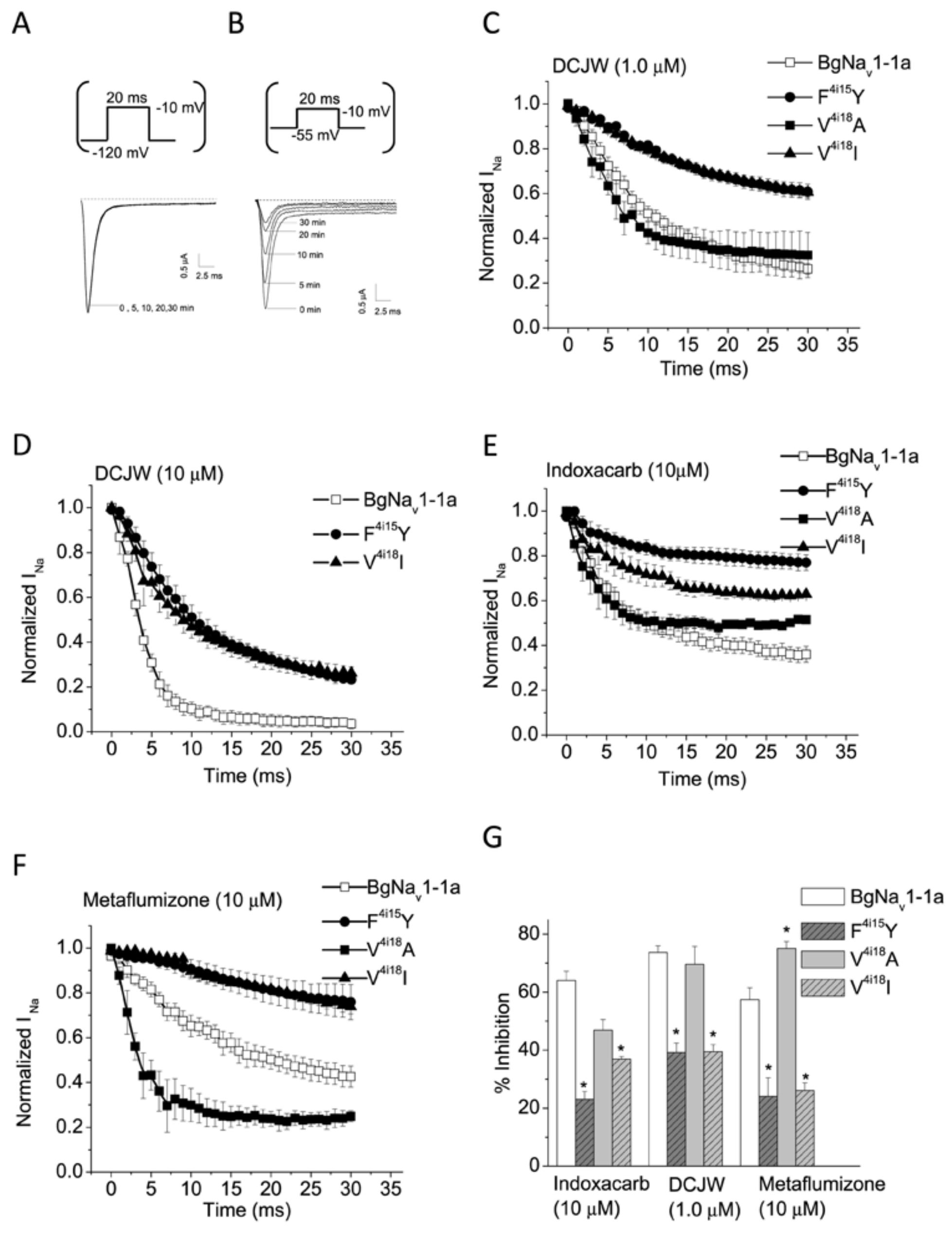

Figure 4.

Time course of inhibition of BgNa$_v$1-1a, F^{4i15}Y, and V^{4i18}A/I sodium channels by indoxacarb, DCJW, and metaflumizone. **A** and **B**. Representative BgNa$_v$1-1a currents recorded with test pulses to -10 mV from the hyperpolarizing holding potential of -120 mV (A) or the depolarizing holding potential of -55 mV (B) at different time points in the presence of 1 µM DCJW. The recording trace immediately preceding the sodium currents was a large capacity current which is not shown and the baseline is indicated with a dash line. **C** and **D**. Inhibition of peak sodium currents by 1 µM (C) and 10 µM DCJW (D). **E** and **F**. Inhibition of peak sodium currents by 10 µM indoxacarb and 10 µM metaflumizone. To measure the inhibition of peak current by SCBIs, test pulses (20 ms) to -10 mV from a depolarizing holding potential (-55 mV for $BgNa_v1$-1a and −60 mV for $F^{4i15}Y$ and $V^{4i18}A/I$ channels) were given once every minute to record the remaining sodium current. The remaining sodium current was then normalized to the current measured prior to application of insecticide. Reduction in "Normalized I_{Na}" reflects the progress of channel inhibition by SCBIs.

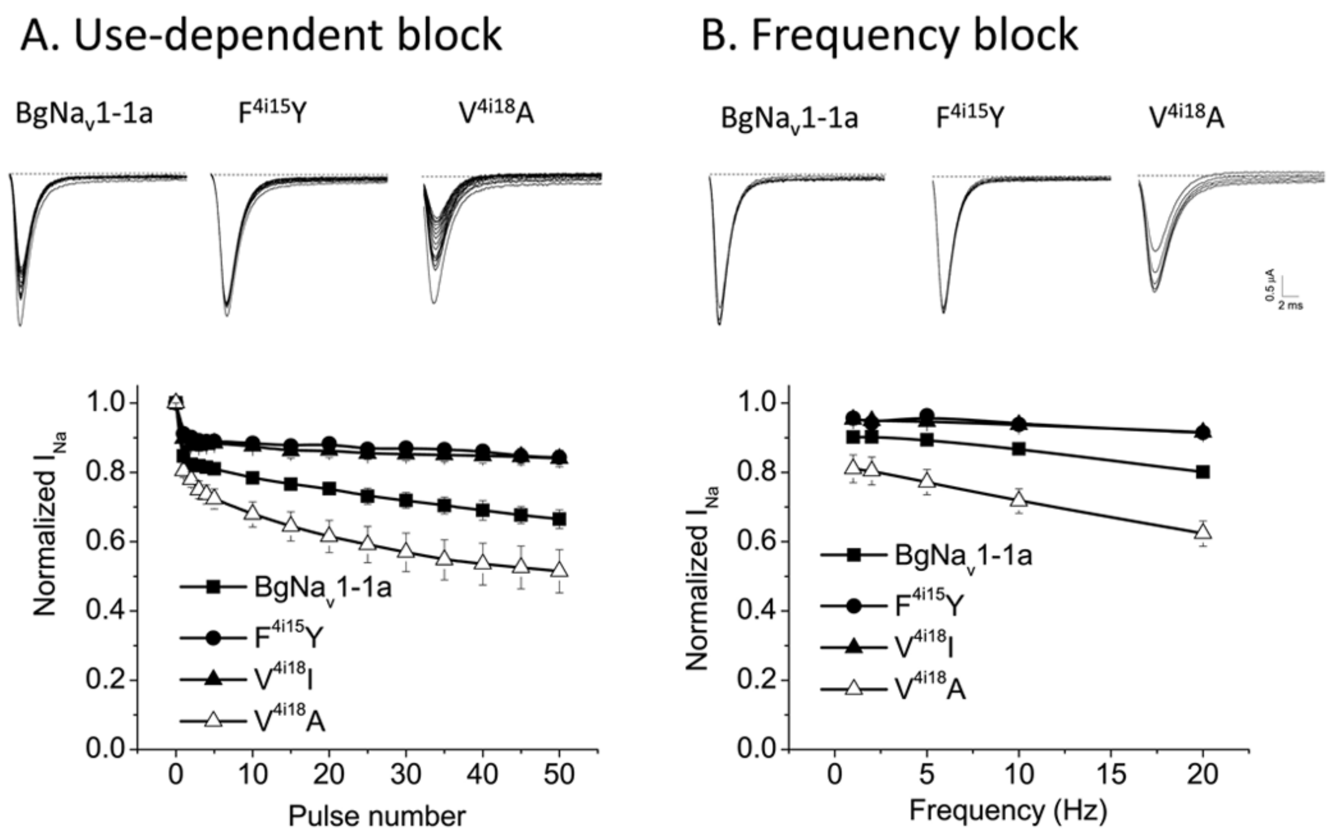

Figure 5.

Use-dependent block (A) and frequency-dependent block (B) of wildtype and mutant channels by lidocaine (2 mM).The recording protocol are shown and details are provided under Materials and Methods. The recording trace immediately preceding the sodium currents was a large capacity current which is not shown and the baseline is indicated with a dash line.

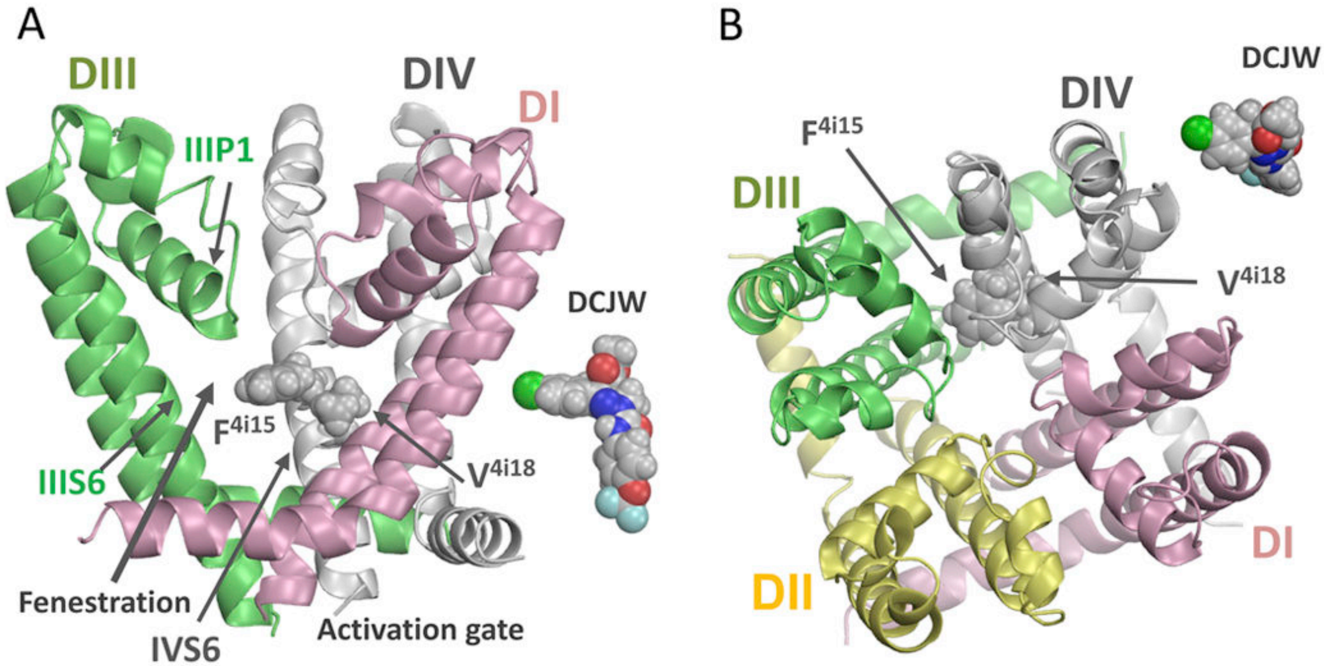

Figure 6.

Side (A) and extracellular (B) views of the pore module in the Na_VAb-based homology model of the insect sodium channel BgNa_V1-1a. Domains DI, DII, DIII, and DIV are shown by pink, yellow, green, and gray ribbons, respectively. Domain DII is removed at the side view for clarity. Side chains of residues F^{4i15} and V^{4i18} are space-filled. For comparison, "side" and "top" views of a DCJW conformer are placed next to respective views of the channel. Mutations F^{4i15}Y and V^{4i18}I enlarge respective residues suggesting that DCJW binds tightly in the inner pore, forms close contacts with F^{4i15} and V^{4i18}, and may expose its hydrophobic moiety to F^{4i15}.

Table 1

Voltage-dependence of activation, fast and slow inactivation of $BgNa_v1$-1a and mutant channels at the holding potential of -120 mV.

	Activation		Fast Inactivation		Slow Inactivation	
	$V_{1/2}$ (mV)	***k***	**$V_{1/2}$ (mV)**	***k***	**$V_{1/2}$ (mV)**	***k***
$BgNa_v1$-1a	-28.2 ± 0.5	5.1 ± 0.9	-49.0 ± 0.5	4.2 ± 0.1	-54.6 ± 0.9	4.2 ± 0.2
$F^{4i15}Y$	-30.7 ± 1.3	5.4 ± 0.4	-48.4 ± 0.5	4.0 ± 0.1	-58.5 ± 1.3	5.5 ± 0.3
$V^{4i18}I$	-27.4 ± 1.4	5.4 ± 0.6	-46.8 ± 0.5	4.1 ± 0.1	-56.7 ± 1.5	3.9 ± 0.2
$V^{4i18}A$	-33.6 ±1.5	6.3 ± 0.9	-50.0 ± 1.1	4.9 ± 0.5	60.7 ± 0.4	3.7 ± 0.3

The voltage dependences of conductance and inactivation were fitted with a two-state Boltzmann equation to determine $V_{1/2}$, the voltage for half- maximal conductance or inactivation, and k, the slope factor for conductance or inactivation. The values in the table represent the mean ± S.E.M. and the number of oocytes was 6-13.

Table 2

Percentage of inhibition of $BgNa_v1$-1a and mutant channels by indoxacarb (10 μM), DCJW (1 and 10 μM) and metaflumizone (10 μM) at the end of 30 min insecticide exposure.

	Indoxacarb		DCJW		Metaflumizone
	10.0	**0.1**	**1.0**	**10.0**	**10.0**
$BgNa_v1$-1a	64.0 ± 3.2	39.0 ± 4.2	73.7 ± 2.3	ND	57.4 ± 4.1
$F^{4i15}Y$	23.0 ± 2.8*	ND	39.2 ± 3.3*	76.7 ± 1.3	24.1 ± 6.4*
$V^{4i18}I$	36.9 ± 0.9*	ND	39.5 ± 2.4*	73.8 ± 2.2	26.1 ± 2.6*
$V^{4i18}A$	46.9 ± 3.7	ND	69.6 ± 6.2	ND	75.3 ± 3.1*

The values of percentage of inhibition were determined by comparing values of “normalized I_{Na}” of channels treated with insecticide to untreated channels at the end of the 30 minutes recording period (See Fig.3). The values in the table represent the mean ± S.E.M. and the number of oocytes was 4-10. The asterisks indicate significant differences from the $BgNa_V1$-1a channel as determined by one-way ANOVA ($p<0.05$) with Scheffe’s post hoc analysis. ND: not determined.