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Three mutations identified in the voltage-sensitive sodium channel α -subunit gene of permethrin-resistant human head lice reduce the permethrin sensitivity of house fly Vssc1 sodium channels expressed in *Xenopus* oocytes

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Abstract

Point mutations in the *para*-orthologous sodium channel α -subunit of the head louse (M815I, T917I and L920F) are associated with permethrin- and DDT-resistance. These mutations were inserted in all combinations using site-directed mutagenesis at the corresponding amino acid sequence positions (M827I, T929I and L932F) of the house fly *para*-orthologous voltage-sensitive sodium channel α -subunit (*Vssc1*^{WT}) gene and heterologously co-expressed with the sodium channel auxiliary subunit of house fly (*Vssc β*) in *Xenopus* oocytes. The double mutant possessing M827I and T929I (*Vssc1*^{MITI}/*Vssc β*) caused a ~4.0 mV hyperpolarizing shift and the triple mutant, *Vssc1*^{MITILF}/*Vssc β* , caused a ~3.2 mV depolarizing shift in the voltage dependence of activation curves. *Vssc1*^{MITI}/*Vssc β* , *Vssc1*^{TILF}/*Vssc β* and *Vssc1*^{MITILF}/*Vssc β* caused depolarizing shifts (~6.6, ~7.6 and ~8.8 mV, respectively) in the voltage dependence of steady-state inactivation curves. The M827I and L932F mutations reduced permethrin sensitivity when expressed alone but the T929I mutation, either alone or in combination, virtually abolished permethrin sensitivity. Thus, the T929I mutation is the principal cause of permethrin resistance in head lice. Comparison of the expression rates of channels containing single, double and triple mutations with that of *Vssc1*^{WT}/*Vssc β* channels indicates that the M827I mutation may play a role in rescuing the decreased expression of channels containing T929I.

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²Abbreviations used: DDT, dichlorodiphenyltrichloroethane; *kdr*, knockdown resistance; PCR, polymerase chain reaction; *Vssc1*, voltage-sensitive sodium channel α -subunit from house fly; *Vssc β* , voltage-sensitive sodium channel auxiliary subunit from house fly.

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Keywords

Heterologous expression; Human head lice; Knockdown resistance; *Pediculus humanus capitis*; Permethrin resistance; Voltage-sensitive sodium channel

1. Introduction

Over the last two decades, resistance to pyrethrins and pyrethroid insecticides by the human head louse, *Pediculus humanus capitis*, has been widely documented (Burgess *et al.*, 1995; Downs *et al.*, 1999a; Downs *et al.*, 1999b; Chosidow *et al.*, 1994; Mumcuoglu *et al.*, 1995; Hemingway *et al.*, 1999; Rupes *et al.*, 1995; Picollo *et al.*, 1998; Vassena *et al.*, 2003; Gao *et al.*, 2003; Yoon *et al.*, 2003; Yoon *et al.*, 2004; Lee *et al.*, 2000b). Heightened public and governmental concerns have occurred because of increased incidents of head louse infestations among school children (<http://www.cdc.gov/ncidod/dpd/parasites/lice/default.htm>; <http://www.headlice.org>). Lee *et al.*, (2000b) first reported that head lice from Massachusetts and Florida were resistant to a pyrethroid, permethrin, and exhibited *in vivo* responses in behavioral bioassays that are consistent with knockdown resistance. Knockdown resistance (*kdr*) is a heritable trait associated with nerve insensitivity to DDT, the pyrethrins and the pyrethroids, that was first discovered in house fly, *Musca domestica* (Busvine, 1951; Farnham, 1977). Phenotypically similar *kdr*-like traits have been subsequently identified in other species of resistant insects (Oppenoorth, 1985; Soderlund, 1997). Genetic analyses determined that these traits are tightly linked to *para*-orthologous voltage-sensitive sodium channel α -subunit genes (Knipple *et al.*, 1994; Williamson *et al.*, 1993; Taylor *et al.*, 1993; Dong and Scott, 1994). Point mutations in these genes are functionally responsible for the *kdr*, *kdr*-like and super-*kdr* traits and nerve insensitivity to DDT, the pyrethrins and pyrethroids (Soderlund and Knipple, 2003).

Three point mutations located in the domain IIS1-2 extracellular loop (M815I) and in the domain IIS5 transmembrane segment (T917I and L920F) of voltage-sensitive sodium channel α -subunit (numbered according to the head louse amino acid sequence) have been identified in permethrin-resistant head lice (Lee *et al.*, 1999a; Lee *et al.*, 2003). All three mutations are found *en bloc* as a haplotype in permethrin-resistant field populations of head louse (Lee *et al.*, 2003). T917I, corresponding to T929I in the house fly, has been functionally validated as a *kdr*-type mutation in the diamondback moth, *Plutella xylostella* (Schuler *et al.*, 1998). It is not yet clear, however, whether the two novel mutations (M815I and L920F) found in head lice have functional significance in pyrethroid resistance.

Heterologous expression of insect voltage-sensitive sodium channels in *Xenopus* oocytes has been utilized to determine the functional characteristics and pharmacological significance of *kdr*-associated point mutations (Lee *et al.*, 2000a; Feng *et al.*, 1995; Smith *et al.*, 1997; Warmke *et al.*, 1997; Tan *et al.*, 2002b; Smith *et al.*, 1998; Zhao *et al.*, 2000; Vais *et al.*, 2000; Lee *et al.*, 1999b). Studies with the wildtype house fly Vssc1 channel coexpressed either with the *Drosophila melanogaster* tipE auxiliary subunit (Vssc1^{WT}/tipE channels) or the house fly Vssc β auxiliary subunit (Vssc1^{WT}/Vssc β channels) in oocytes and with mutated Vssc1 variants carrying the *kdr*-associated L1014F, M918T and V410M mutations demonstrated that the single (L1014F) or double (L1014F and M918T) *kdr*-type mutations significantly reduced or abolished pyrethroid sensitivity (Smith *et al.*, 1997; Lee *et al.*, 1999b; Lee and Soderlund, 2001). This system has also been employed to characterize the functional significance of the *kdr*-type mutation (V410M) found in another insect species, *Heliothis virescens* (Lee and Soderlund, 2001).

In the present study we employed site-directed mutagenesis to insert the three mutations associated with pyrethroid resistance in the head louse (M815I, T917I and L920F) in all possible combinations into the corresponding positions of the *Vssc1*^{WT} sequence, expressed wildtype and specifically-mutated channels in *Xenopus* oocytes, and employed electrophysiological assessments to characterize the impact of these mutations on the expression, gating properties and permethrin sensitivity of the expressed channels.

2. Materials and Methods

2.1. Site-directed mutagenesis and construction of cDNA clones

The house fly voltage-sensitive sodium channel α -subunit cDNA (*Vssc1*^{WT}) in palter[®]-1 (*Vssc1*^{WT}-pAlter) (Promega, Madison, WI) (Smith *et al.*, 1997; Lee *et al.*, 2000a) was used to prepare a short cDNA fragment in order to perform the PCR-based mutagenesis. This cDNA clone contained exon k in IIS3-IIS4 making it less sensitive to pyrethroids when expressed than these clones containing exon l (Lee *et al.*, 2002; Tan *et al.*, 2002a). A 2.3 kb fragment (0.5–1.0 μ g) of *Vssc1*^{WT} (*Mut*^{WT}) was obtained using XbaI restriction digestion, separated by agarose gel (1 %, w/v) electrophoresis and purified using QIAEX[®] II Gel Extraction Kit (Qiagen Inc.) following manufacturer's instruction. The pGEM[®]-3Z vector (1 μ g, Promega) was linearized with XbaI (20 U), followed by purification using Microcon[®] filter (Millipore Corp., Bedford, MA) and electrophoresed (1 %, w/v, agarose gel) to confirm linearization and to determine the final concentration using a DNA mass ladder (Invitrogen, Carlsbad, CA). Approximately 0.13 μ g of *Mut*^{WT} and 0.05 μ g of the linearized pGEM[®]-3Z vector (3:1 molar ratio) were ligated using T4 DNA ligase (400U, New England Biolabs, Inc.) at 16 °C for 30 min, followed by heat inactivation at 65 °C for 10 min. Transformation of 100 μ l JM109 competent cells (Promega) with the ligation product (5–10 ng) was performed following manufacturer's instruction.

Mutations corresponding to M815I, T917I and L920F in the head louse sodium channel sequence were inserted into the corresponding positions (M827I, T929I and L932F, respectively) of *Mut*^{WT} individually and in combination by site-directed mutagenesis. Mutagenic oligonucleotide primer sets (Table 1) were designed based on the sequence of *Vssc1*^{WT}. Mutated plasmid DNAs were synthesized by PCR (one cycle of 95 °C for 1 min, followed by 18 cycles of 95 °C for 50 sec, 60 °C for 50 sec, 68 °C for 5 min 10 sec, followed by one cycle of 68 °C for 7 min) using the QuikChange[®] II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the *Mut*^{WT}-pGEM[®]-3Z (0.01 μ g) with a specific set of mutagenic oligonucleotides (0.125 μ g/oligonucleotide). Restriction enzyme DpnI (10 U, Stratagene) was added to digest methylated and hemimethylated template DNA by incubation at 37 °C for 60 min. XL10-Gold[®] competent cells (45 μ l, Stratagene), pretreated with 2 μ l β -mercaptoethanol, were transformed using 2 μ l of the mutagenesis products to give the three singly-mutated constructs (designated *Mut*^{MI}-, *Mut*^{TI}- and *Mut*^{LF}-pGEM[®]-3Z, respectively).

Double (*Mut*^{MITI}-, *Mut*^{MILF}- and *Mut*^{TILF}-pGEM[®]-3Z) and triple (*Mut*^{MITILF}-pGEM[®]-3Z) mutant DNAs were subsequently synthesized as described above. For example, *Mut*^{MI}-pGEM[®]-3Z and primers (5fly-TI and 3fly-TI) were used to synthesize *Mut*^{MITI}-pGEM[®]-3Z. Once all double mutant DNAs were synthesized, *Mut*^{MITILF}-pGEM[®]-3Z was synthesized using *Mut*^{MITI}-pGEM[®]-3Z and 5fly-LF and 3fly-LF primers. The presence of mutations were confirmed by DNA sequencing analysis (ABI 377XL, Applied Biosystems, Foster City, CA).

Mutated fragments (*Mut*^{MI}, *Mut*^{TI}, *Mut*^{LF}, *Mut*^{MITI}, *Mut*^{MILF}, *Mut*^{TILF} and *Mut*^{MITILF}) were cut from pGEM[®]-3Z by XbaI digestion, agarose gel-purified and ligated into XbaI-digested *Vssc1*^{WT}-pAlter to construct full-length mutated *Vssc1*s (designated *Vssc1*^{MI}, *Vssc1*^{TI}, *Vssc1*^{LF}, *Vssc1*^{MITI}, *Vssc1*^{MILF}, *Vssc1*^{TILF} and *Vssc1*^{MITILF}, respectively). The orientation and

size of inserted mutated fragments were verified by diagnostic colony PCRs, restriction digestions and DNA sequencing.

2.2. Heterologous expression of *Vssc1s* in *Xenopus* oocytes

Plasmid DNAs (~2–3 µg) containing the wildtype (*Vssc1^{WT}*) and mutated *Vssc1s* and *Vsscβ* were linearized using SphI. The synthesis of cRNAs from SphI-digested linearized plasmid DNAs, containing T7 promoter 5' upstream of the *Vssc1s* and *Vsscβ*, were individually carried out using mMESSAGE mMACHINE® *in vitro* transcription kit (Ambion, Austin, TX) following the manufacturer's instructions. The integrity and size of the cRNAs were determined by 1 % agarose-2.2 M formaldehyde gel electrophoresis with ethidium bromide staining.

Oocytes were surgically removed from a female *Xenopus laevis* (Nasco, Ft. Atkinson, WI), which had been fully anesthetized with 0.2 % (w/v) ethyl 3-aminobenzoate methanesulfonate (Sigma, St. Louis, MO) dissolved in water, and defolliculated by incubation (~30–45 min) in OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂ and 5 mM HEPES, pH 7.5) supplemented with Type 1A collagenase (0.5–1.0 U/ml, Sigma). The remaining follicle membrane was removed manually with fine forceps. Isolated stage V–VI oocytes were incubated in ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES, pH 7.4) supplemented with 1 % sodium pyruvate, 1 % penicillin/streptomycin and 5 % horse serum overnight at 19 °C prior to cRNA injection (Goldin, 1992; Soreq and Seidman, 1992). At least 3–5 different batches of oocytes were co-injected with 60–90 nl of a mixture containing 1.0 ng *Vssc1s*/nl and 0.33 ng *Vsscβ*/nl cRNAs using a Nanoliter 2000 nanoinjector (World Precision Instruments, Sarasota, FL) and incubated in supplemented ND-96 medium at 19 °C for 2–5 days prior to electrophysiological recordings.

2.3. Electrophysiological recordings and data analysis

Membrane currents were obtained from oocytes maintained in ND-96 at 22–24 °C using a two-microelectrode voltage clamp (TEVC) system (GeneClamp 500B, Axon Instruments, Inc., Foster City, CA) with a virtual ground (VG-2A, Axon Instruments, Inc.). A 250 µl bath (15 × 2.0 mm, diameter × depth) was fabricated from a silicone-chambered coverslip (CulterWell™, Sigma) that was firmly affixed onto a glass slide (75 × 25 mm). Voltage and current microelectrodes (0.5–2.0 MΩ) were made using borosilicate glass capillaries and filled with 3 M KCl. The barrel of voltage microelectrode was coated with silver conductive paint (CW2200STP, ITW Chemtronics Inc., Kennesaw, GA), the circular boundary between the barrel and the shank of voltage microelectrode was overcoated with insulating resin (CW3300, ITW Chemtronics Inc.), and the barrel shielded with grounded aluminum foil. Data were filtered with a 2 kHz low-pass (4-pole Bessel) filter, digitized at 20 kHz using a digitizer (Digidata 1322A, Axon Instruments, Inc.) and stored by pClamp software (ver. 8.2, Axon Instruments Inc.). Net sodium currents were obtained by subtracting traces from the same oocyte in the presence of 20 µM tetrodotoxin (TTX, Sigma).

Percent expression values were calculated using Equation 1 (Eq. 1) to determine whether individual and combinations of mutations reduced expression rate. To minimize experimental variability, data was collected from oocytes injected with all variants of cRNAs (*Vssc1^{WT}*/*Vsscβ*, *Vssc1^{MI}*/*Vsscβ*, *Vssc1^{TI}*/*Vsscβ*, *Vssc1^{LF}*/*Vsscβ*, *Vssc1^{MITI}*/*Vsscβ*, *Vssc1^{MILF}*/*Vsscβ*, *Vssc1^{TILF}*/*Vsscβ* and *Vssc1^{MITILF}*/*Vsscβ*, respectively) on the same day of recording.

$$\% \text{ Expression} = \frac{\text{Number Expressed}}{\text{Number Injected}} \times 100 \quad \text{Eq. 1)}$$

Where *Number Expressed* = number of oocytes producing TTX-sensitive sodium currents (> 200 nA).

Number Injected = total number of oocytes co-injected with the same amount of *VsscI* and *Vsscβ* cRNAs.

The voltage dependence of activation was determined by the amplitude of peak transient sodium currents obtained upon a 50 ms depolarization from a holding potential of −100 mV to test potential ranging from −100 to 50 mV (in 5 mV step increments). The peak transient sodium currents were converted to the sodium conductance (*G*) values using Equation 2 (Eq. 2):

$$G = I_{peak} / (V_t - V_{rev}) \quad \text{Eq. 2}$$

Where *I_{peak}* = current amplitude at each test potential.

V_t = test membrane potential.

V_{rev} = reversal potential for sodium current.

The sodium conductance values (*G*) were normalized to the maximal sodium conductance (*G_{max}*) and fitted with a Boltzmann equation for the voltage dependence of activation (Equation 3):

$$G = \frac{G_{max}}{[1 + \{\exp(V_t - V_{0.5})/k\}]} \quad \text{Eq. 3}$$

Where *V_t* = test membrane potential.

V_{0.5} = half maximal potential at which *G* = 0.5.

k = slope factor.

The voltage dependence of steady-state inactivation was determined using a two-pulse protocol that began with a voltage-step from a holding potential of −100 mV to a conditioning prepulse potential ranging from −100 to 50 mV (in 5 mV step increments) for 160 ms, followed by a 30 ms test pulse from −100 to 20 mV after a brief 1 ms step to the holding potential. The peak current amplitude (*I*) values during the test depolarization were normalized to the maximum current amplitude (*I_{max}*) and fitted with a Boltzmann equation for the voltage dependence of steady-state inactivation (Equation 4):

$$I = \frac{I_{max}}{[1 + \{\exp(V_{pp} - V_{0.5})/k\}]} \quad \text{Eq. 4}$$

Where *V_{pp}* = prepulse membrane potential.

V_{0.5} = half maximal potential at which *I* = 0.5.

k = slope factor.

A stock solution of (*IR*)-*cis*-permethrin (E3343-144, 94 % purity, FMC Corp., Philadelphia, PA) dissolved in dimethyl sulfoxide (DMSO, Sigma) was prepared and diluted in ND-96 medium immediately before bath application. Final concentration of DMSO in the bath did not exceed 0.2 % (v/v), a concentration that had no effect on sodium currents. Oocytes were incubated with (*IR*)-*cis*-permethrin (0–200 μM) in a non-perfused recording bath at 22–24 °C for 5 min prior to applying the pre-programmed pulse protocol, a 50 ms depolarization from a holding potential of −100 mV to a −5 mV test potential. Sodium channel modification by

permethrin was determined by comparing three different parameters (normalized maximum tail current amplitude, normalized late current amplitude, and tail current decay constant) measured from the oocytes treated with 200 μ M permethrin to the corresponding values obtained from untreated oocytes. The maximum tail current was determined at the moment of repolarization (ca. 51 ms) if it was monophasic, or at the peak if biphasic. The late current was defined as the prolonged currents during depolarization (Smith *et al.*, 1997), and measured at the end of 50 ms depolarization. The amplitudes of both tail and late currents induced by permethrin were normalized to the amplitude of peak transient current measured in the same oocyte prior to permethrin application. The tail current decay constant (τ) was determined from the falling phase of tail current by fitting to a single exponential.

3. Results

3.1. Effects of MI, TI and LF mutations alone and in combination on the expression of Vssc1/Vssc β sodium channels in *Xenopus* oocytes

Tetrodotoxin (TTX)-sensitive peak transient sodium currents were obtained in oocytes co-injected with a *Vssc1* variant (*Vssc1*^{WT}, *Vssc1*^{MI}, *Vssc1*^{TI}, *Vssc1*^{LF}, *Vssc1*^{MITI}, *Vssc1*^{MILF}, *Vssc1*^{TILF} or *Vssc1*^{MITILF}) and *Vssc β* cRNAs. Expression rates (%) and amplitudes of peak transient currents were compared to determine whether individual or combinations of mutations affected expression (Table 2). The expression of *Vssc1*^{TI}/*Vssc β* , *Vssc1*^{LF}/*Vssc β* and *Vssc1*^{TILF}/*Vssc β* channels was significantly decreased compared to the expression of *Vssc1*^{WT}/*Vssc β* channels. However, the expression of *Vssc1* variants containing the M827I mutation (*Vssc1*^{MI}/*Vssc β* , *Vssc1*^{MITI}/*Vssc β* , *Vssc1*^{MILF}/*Vssc β* and *Vssc1*^{MITILF}/*Vssc β*) did not differ statistically from the expression rate for *Vssc1*^{WT}/*Vssc β* channels.

The mean amplitudes of peak transient sodium currents measured in oocytes expressing *Vssc1*^{TI}/*Vssc β* and *Vssc1*^{TILF}/*Vssc β* channels were significantly reduced compared to those for *Vssc1*^{WT}/*Vssc β* channels (*t*-test, $P < 0.05$). *Vssc1*^{LF}/*Vssc β* and *Vssc1*^{MITI}/*Vssc β* gave mean peak transient currents that were ~1.5-fold greater than those found for *Vssc1*^{WT}/*Vssc β* channels, but these differences were not statistically significant (*t*-test, $P > 0.05$). All remaining *Vssc1* variants gave mean peak transient currents that were not significantly different from those found for *Vssc1*^{WT}/*Vssc β* channels (*t*-test, $P > 0.05$).

3.2. Effects of the MI, TI and LF mutations on voltage-dependent channel gating

To identify effects of mutations on the voltage dependence of activation, we generated families of sodium current traces (Fig. 1A, insert) and converted peak transient sodium currents obtained at different test potentials to conductance values as described in Eq. 2. Conductance values, normalized to the maximum sodium conductance value in each experiment, were plotted against test potential and fitted to the Boltzmann distribution to determine midpoint potentials ($V_{0.5}$) and slope factors (k) (Fig. 1A and Table 3). For *Vssc1*^{MITI}/*Vssc β* channels, the voltage dependence of activation was shifted by ~4.0 mV in the direction of hyperpolarization. In contrast, the activation curve for *Vssc1*^{MITILF}/*Vssc β* channels was shifted by ~3.2 mV in the direction of depolarization. The activation curves for the remaining mutated *Vssc1* variants were not significantly different from that found for *Vssc1*^{WT}/*Vssc β* channels (*t*-test, $P > 0.05$).

To identify effects of mutations on the voltage dependence of steady-state inactivation, we employed a two pulse protocol to generate families of sodium current traces (Fig. 1B, insert). A 1-ms step to the holding potential (−100 mV) was inserted between the conditioning and test pulses to facilitate the subtraction of capacitive currents; this brief repolarization did not significantly affect the measurement of steady-state inactivation. Plots of mean normalized peak currents as a function of conditioning prepulse potential were fitted to the Boltzmann distribution to determine midpoint potentials ($V_{0.5}$) and slope factors (k) (Fig. 1B and Table

3). For $Vssc1^{MITI}/Vssc\beta$, $Vssc1^{TILF}/Vssc\beta$ and $Vssc1^{MITILF}/Vssc\beta$ channels, midpoints for steady-state inactivation were significantly shifted in the direction of depolarization by ~6.6, ~7.6 and ~8.8 mV, respectively). Midpoints for steady-state inactivation of the remaining $Vssc1$ variants were not significantly different from that measured for $Vssc1^{WT}/Vssc\beta$ channels (t -test, $P > 0.05$).

3.3. Effects of the MI, TI and LF mutations on the permethrin sensitivity of $Vssc1^{WT}/Vssc\beta$ sodium channels

Figure 2A shows superimposed sodium current traces obtained from a single oocyte expressing $Vssc1^{WT}/Vssc\beta$ channels before and after exposure to concentrations of permethrin ranging from 0.1 μ M to 200 μ M. These traces illustrate the two characteristic effects of permethrin on sodium currents: production of late currents carried by channels that remain open at the end of a 50-ms depolarization, and production of biphasic tail currents upon repolarization at the end of a depolarizing pulse. In assays with 200 μ M permethrin, the mean normalized tail and late current amplitudes were 0.443 ± 0.158 and 0.224 ± 0.055 , respectively, compared to a mean peak current amplitude prior to permethrin of 1.0 (Table 4). In the presence of 200 μ M permethrin, the mean tail current decay constant (τ) determined from oocytes expressing $Vssc1^{WT}/Vssc\beta$ channels was 545.9 ± 21.4 ms (Table 4). The amplitudes of permethrin-induced tail and late currents carried by $Vssc1^{WT}/Vssc\beta$ channels increased in a concentration-dependent manner (Fig. 2A). Plots of normalized maximum tail and late currents versus permethrin concentration were generated to quantify the effects of the mutations on channel sensitivity to permethrin (Fig. 3).

Each of the three mutations ($Vssc1^{MI}$, $Vssc1^{TI}$ and $Vssc1^{LF}$) alone reduced the permethrin sensitivity of $Vssc1^{WT}/Vssc\beta$ sodium channels to permethrin (Figs. 2B–D). Permethrin-modified sodium currents recorded from oocytes expressing $Vssc1^{MI}/Vssc\beta$ (Fig. 2B) or $Vssc1^{LF}/Vssc\beta$ (Fig. 2D) channels exhibited tail and late currents that were substantially reduced in amplitudes compared to the permethrin-modified currents from oocytes expressing $Vssc1^{WT}/Vssc\beta$ channels (Fig. 2A). Mean data from multiple experiments (Figs. 3A and 3D; Table 4) show that mutation-dependent reductions in tail and late current amplitude for these two variants were particularly evident at 100–200 μ M permethrin. At 200 μ M permethrin, the normalized maximum tail and late currents from amplitudes obtained with $Vssc1^{MI}/Vssc\beta$ and $Vssc1^{LF}/Vssc\beta$ channels were reduced by 2.5- to 3-fold compared to those obtained from $Vssc1^{WT}/Vssc\beta$ channels. In contrast, current traces obtained from oocytes expressing $Vssc1^{TI}/Vssc\beta$ channels in the presence of up to 200 μ M permethrin were indistinguishable from DMSO control current traces recorded prior to permethrin exposure (Figs. 2C, 3A and 3D; Table 4).

Figures 2E–2G illustrate the effects of 0.1–200 μ M permethrin on all three sodium channel constructs containing two of the three resistance-associated mutations. Sodium current traces recorded in the presence of permethrin from oocytes expressing the two doubly-mutated channels that contained the T929I mutation ($Vssc1^{MITI}/Vssc\beta$ and $Vssc1^{TILF}/Vssc\beta$; Figs. 2E and G) were indistinguishable from DMSO control current traces. All permethrin concentrations (0.1–200 μ M) failed to significantly alter the tail and late currents in oocytes expressing these variants (Fig. 3B, 3E and Table 4). Whereas $Vssc1^{MILF}/Vssc\beta$ channels also exhibited markedly reduced sensitivity to permethrin, permethrin-induced late and tail currents were still clearly detectable at the highest permethrin concentrations (Figs. 2F, 3B and 3E). At 200 μ M permethrin, the normalized maximum tail and late currents from amplitudes obtained with $Vssc1^{MILF}/Vssc\beta$ channels were reduced by 6.8- and 3.6-fold, respectively, compared to those obtained from $Vssc1^{WT}/Vssc\beta$ channels (Table 4).

Figure 2H illustrates the effects of 0.1–200 μ M permethrin on the triply-mutated $Vssc1^{MITILF}/Vssc\beta$ channels which mimic the combination of sodium channel mutations

found in permethrin-resistant louse populations. Superimposed current traces obtained 5 min after permethrin treatments were indistinguishable from DMSO control traces recorded prior to permethrin treatments. Like all other channel variants containing the T929I mutation, the Vssc1^{MILF}/Vssc β channel was virtually insensitive to permethrin over the range of permethrin concentrations examined (Figs. 3C and 3F).

The Vssc1^{MI}/Vssc β , Vssc1^{LF}/Vssc β and Vssc1^{MILF}/Vssc β variants, all of which exhibited reduced but still detectable sensitivity to permethrin based on the reduction of tail and late current amplitudes, also exhibited accelerated decay of permethrin-induced tail currents. Based on mean first-order decay constants (τ values) obtained from channels exposed to 200 μ M permethrin, the tail currents obtained with the Vssc1^{MI}/Vssc β and Vssc1^{LF}/Vssc β variants decayed 3.5- and 4.1-fold more rapidly than those observed for permethrin-modified Vssc1^{WT}/Vssc β channels (Table 4). Similarly, tail currents obtained with the Vssc1^{MILF}/Vssc β decayed 7.8-fold more rapidly than those observed for permethrin-modified Vssc1^{WT}/Vssc β channels (Table 4). For these three channel variants, the degree of acceleration of tail current decay paralleled the degree of reduction in permethrin sensitivity based on the normalized amplitudes of permethrin-induced late and tail currents obtained from oocytes exposed to 200 μ M permethrin (Table 4). In all cases in which permethrin produced detectable tail currents, the rate constants for tail current decay did not vary with permethrin concentration (see Figs. 2A, 2B, 2D and 2E).

4. Discussion

Several studies have employed the functional expression in *Xenopus* oocytes of the house fly Vssc1 sodium channel α subunit in combination with sodium channel auxiliary subunits from either *D. melanogaster* (tipE) or the house fly (Vssc β) to characterize the actions of pyrethroid insecticides and assess the functional significance of sodium channel gene mutations that are associated with pyrethroid resistance. These studies have documented two distinct types of modification of Vssc1 sodium channels by pyrethroids that are dependent on the structure of the pyrethroid employed. The majority of studies have employed cismethrin (Smith *et al.*, 1997; Smith *et al.*, 1998; Lee *et al.*, 1999b; Lee and Soderlund, 2001). Cismethrin, a non- α -cyano-containing and a T-syndrome-producing pyrethroid, appears to bind predominantly to the resting state of Vssc1^{WT}/tipE channels and produces prolonged late currents and biphasic tail currents when assayed under voltage clamp conditions. Repeated depolarizations do not enhance cismethrin modification, indicating that binding to open channels either does not occur or is not preferred over binding to closed channels. In contrast, cypermethrin, classified as an α -cyano-containing and CS-syndrome-producing pyrethroid, causes no detectable first-pulse (i.e., resting state) modification of Vssc1^{WT}/tipE channels. Instead, cypermethrin causes profound use-dependent modification of sodium currents with more persistent tail currents than those induced by cismethrin (Smith *et al.*, 1998; Lee *et al.*, 1999b). Studies with deltamethrin, a close structural relative of cypermethrin, and other insect sodium channels in the *Xenopus* oocyte expression system confirm the exclusively use-dependent action of these α -cyano pyrethroids (Vais *et al.*, 2000; Tan *et al.*, 2002b; Vais *et al.*, 2003).

Previous investigations by other groups on the effects of permethrin, nominally a non-cyano/T-syndrome pyrethroid, on insect sodium channels expressed in oocytes suggest that this compound is able to interact with the resting state (Warmke *et al.*, 1997; Zhao *et al.*, 2000) and both the resting and open states of *D. melanogaster* Para/tipE sodium channels (Vais *et al.*, 2003). Permethrin-dependent channel modification is evident upon the first depolarization after permethrin exposure (Warmke *et al.*, 1997; Zhao *et al.*, 2000), but permethrin-dependent modification is enhanced upon repeated depolarization or co-exposure to sea anemone toxin II, which selectively blocks inactivation and creates persistently open channels (Vais *et al.*, 2003). In our study, permethrin treatments on Vssc1^{WT}/Vssc β channels without prepulses (i.e.

channels in the resting state) produced large amplitudes of late and tail currents, providing further evidence for the ability of permethrin to modify insect sodium channels in the resting state, although it remains to be elucidated which state of channel is more preferentially modified by permethrin. Moreover, the effects of permethrin on Vssc1^{WT}/Vsscβ channels in the present study were qualitatively identical to the effects described previously of cismethrin on Vssc1^{WT}/tipE channels in this system (Smith *et al.*, 1997; Smith *et al.*, 1998; Lee *et al.*, 1999b; Lee and Soderlund, 2001).

The three point mutations (M815I, T917I and L920F, amino acid number based on head louse) associated with permethrin- and DDT-resistance in the human head louse (Lee *et al.*, 2003) each reduced the sensitivity of Vssc1^{WT}/Vsscβ sodium channels to permethrin when inserted by mutagenesis at the corresponding positions (M827I, T929I and L932F) of the Vssc1^{WT} sequence. Our studies with the singly-mutated M827I and L932F variants provide the first information on the impact of these mutations on the sensitivity of insect sodium channels to pyrethroids. Both of these mutations reduce the pyrethroid sensitivity of the expressed channels, as measured by reductions in normalized tail current amplitude. Assays with channels containing the M827I and L932I mutations, either singly or in combination, also exhibited acceleration of the rate of tail current decay that was correlated with the degree of resistance afforded to 200 μM permethrin. Both the reduction in permethrin sensitivity and the acceleration of tail current decay observed with channels containing the M827I and L932I mutations are qualitatively similar to the previously-characterized effects of the V410M and L1014F mutations on the permethrin sensitivity of *D. melanogaster* Para/tipE sodium channels (Lee *et al.*, 2000a; Feng *et al.*, 1995; Smith *et al.*, 1997; Warmke *et al.*, 1997; Tan *et al.*, 2002b; Smith *et al.*, 1998; Zhao *et al.*, 2000; Vais *et al.*, 2000; Lee *et al.*, 1999b) and the cismethrin sensitivity of Vssc1^{WT}/tipE sodium channels (Lee *et al.*, 2000a; Feng *et al.*, 1995; Smith *et al.*, 1997; Warmke *et al.*, 1997; Tan *et al.*, 2002b; Smith *et al.*, 1998; Zhao *et al.*, 2000; Vais *et al.*, 2000; Lee *et al.*, 1999b).

Although the degree of acceleration of tail current decay in Vssc1^{MI}, Vssc1^{LF} and Vssc1^{MILF} channels was tightly correlated with the degree of permethrin resistance afforded by these mutations, the mechanistic relationship between resistance and altered tail current decay is not clear. The decay constants for permethrin-induced tail currents did not vary with permethrin concentration, implying that the rate of tail current decay is an intrinsic property of each combination of pyrethroid and channel variant. Moreover, studies of the action of deltamethrin on pyrethroid resistance mutations in German cockroach sodium channels showed that resistance in several different mutated channel variants was not correlated with the acceleration of the decay of deltamethrin-induced tail currents (Tan *et al.*, 2002b). Therefore, changes in the rate of tail current decay cannot be regarded as reliable indices of changes in the pyrethroid sensitivity of insect sodium channels.

The M827I and L932I point mutations are widely separated in homology domain II of the Vssc1 sodium channel protein based on predicted topology. The L932F mutation occurs at a residue located in the inner pore region of the channel that has been implicated, on the basis of structural and ligand-docking models, as a component of the pyrethroid receptor site of the house fly sodium channel (O'Reilly *et al.*, 2006). Our data therefore provide important confirmation of the significance of this residue in determining the pyrethroid sensitivity of sodium channels. In contrast, the M827I mutation is located on an extracellular segment of homology domain II, a region of the channel not envisioned as being directly involved in pyrethroid binding (O'Reilly *et al.*, 2006). It is therefore likely that the M827I mutation alters pyrethroid sensitivity indirectly by changing the conformation of the pyrethroid receptor or by altering the gating properties of the channel to impair pyrethroid modification. Nevertheless, we did not identify effects of this mutation on channel gating (see Table 3) that might account for its effect on permethrin sensitivity. Our data also suggest that the M827I and L932F

mutations exert independent effects on channel sensitivity because the properties of the doubly-mutated channel approximate the expected additive effects of the two mutations expressed individually.

In contrast to the M827I and L932F mutations, T929I mutation produced profound and unexpected effects on the sensitivity of $V_{ssc1}^{WT}/V_{ssc\beta}$ sodium channels to permethrin. The T929I mutation has been identified in pyrethroid-resistant diamondback moth, *Plutella xylostella*, and shown to be the causative mutation for *kdr*-like resistance in that species (Schuler *et al.*, 1998). A previous study of the impact of the T929I mutation on the permethrin sensitivity of Para/tipE sodium channels showed that this mutation conferred a 13-fold decrease in sensitivity (Vais *et al.*, 2003). In contrast, we found that the T929I mutation virtually abolished the permethrin sensitivity of $V_{ssc1}^{WT}/V_{ssc\beta}$ sodium channels whether present as a single mutation or in combination with one or both of the other resistance-associated mutations.

There are two possible explanations for the divergent effects of the T929I mutation on permethrin sensitivity in these two studies. First, this mutation could exert differential effects on the sensitivity of resting and open channels to permethrin. Our study focused exclusively on resting modification, whereas the previous study employed methods (including the use of sea anemone toxin II to enhance the availability of open channels) that were biased toward the observation of open channel modification (Vais *et al.*, 2003). A selective effect of the T929I mutation on the sensitivity of resting channels to permethrin modification would be consistent with the results obtained in both studies. Second, the effects of the T929I mutation may depend on the sodium channel sequence context in which it is expressed. We examined the effects of this mutation in the house fly V_{ssc1} sodium channel, whereas the previous study employed the *D. melanogaster* para sodium channel as the template for mutagenesis. Despite these differences, the results of both studies are consistent with the identification of this residue as one of the elements of the pyrethroid receptor on insect sodium channels (O'Reilly *et al.*, 2006).

In light of the powerful effect of the T929I mutation by itself on permethrin sensitivity, it is surprising that resistant louse populations have only been found to contain all three resistance-associated mutations. The situation in head louse may be comparable to *super-kdr* trait in house fly (Lee *et al.*, 1999b; Soderlund and Knipple, 2003). Two house fly mutations (M918T and L1014F) cause complete insensitivity to cismethrin and cypermethrin, whereas L1014F alone elicits only ~10-fold reduced sensitivity to cismethrin. Single mutant channels carrying only M918T express a high level of cismethrin insensitivity, but this single mutation also severely impaired the expression of functional V_{ssc1} sodium channels. It was suggested that the double mutation in pyrethroid-resistant house flies is necessary due to the severe impairment of V_{ssc1} function by M918T and that this is somehow complemented by the L1014F mutation. Similarly, T929I reduces the expression rate of $V_{ssc1}^{WT}/V_{ssc\beta}$ sodium channels in oocytes (Table 2). Moreover the presence of the M827I mutation in combination with T929I rescues the deleterious impact of the T929I mutation in the V_{ssc1}^{MITI} and V_{ssc1}^{MITILF} channel variants. This analysis, however, does not account for the occurrence of the L932F mutation in the resistance associated louse haplotype.

The issue of fitness costs of resistance mutations is important to understanding the selection and persistence of resistant phenotypes in the field. For example, Zhao *et al.*, (2000) reported that the depolarizing shift in the voltage-dependent activation of sodium channels in pyrethroid-resistant *Heliothis virescens* carrying the point mutations V410M and L1014F (amino acid number based on house fly) was associated with reduced neuronal excitability and sluggish behavior, providing the basis for its reduced fecundity and mating success. Takano-Lee *et al.*, (2003) and Yoon *et al.*, (2006), however, have reported that there is no major fitness disadvantage in the permethrin- and DDT-resistant head louse strain (SF-HL), suggesting that

the resistant lice have evolved means to minimize any fitness disadvantage associated with resistance mutations. Because the three mutations exist *en bloc* as a DDT- and permethrin-resistant haplotype, the two novel mutations (M815I and L920F) may function to compensate for any fitness disadvantage associated with the T917I mutation in permethrin- and DDT-resistant head lice.

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Figure 1. Voltage dependence of activation of $Vssc1^{WT}/Vssc\beta$, $Vssc1^{MITI}/Vssc\beta$ and $Vssc1^{MITILF}/Vssc\beta$ expressed in *Xenopus* oocytes (A). Mean normalized conductance values were transformed from current-voltage data obtained from 3-10 separated experiments. Insert: A family of representative sodium current traces obtained from an oocyte expressing $Vssc1^{WT}/Vssc\beta$. Voltage dependence of steady-state inactivation of $Vssc1^{WT}/Vssc\beta$, $Vssc1^{MITI}/Vssc\beta$, $Vssc1^{TILF}/Vssc\beta$ and $Vssc1^{MITILF}/Vssc\beta$ expressed in *Xenopus* oocytes (B). Mean normalized current values were obtained from 3-6 separated experiments. Insert: A family of representative sodium current traces obtained from an oocyte expressing $Vssc1^{WT}/Vssc\beta$.

Figure 2.

Comparative sodium current traces obtained before and after exposure to permethrin (black for DMSO control; cyan for 0.1 μM permethrin; magenta for 1.0 μM permethrin; blue for 10 μM permethrin; green for 100 μM permethrin; red for 200 μM permethrin) from *Xenopus* oocytes expressing *Vssc1*^{WT}/*Vsscβ* (A), *Vssc1*^{MI}/*Vsscβ* (B), *Vssc1*^{TI}/*Vsscβ* (C), *Vssc1*^{LF}/*Vsscβ* (D), *Vssc1*^{MITI}/*Vsscβ* (E), *Vssc1*^{MILF}/*Vsscβ* (F), *Vssc1*^{TILF}/*Vsscβ* (G) and *Vssc1*^{MITILF}/*Vsscβ* (H). Prior to and 5 min after bath application of permethrin (0.1–200 μM), sodium current traces from individual oocytes were obtained during and after a 50 ms depolarizations from −100 mV to −5 mV.

Figure 3.

Effects of permethrin treatments (0.1–200 μM) on the normalized amplitude of the sodium tail and the late currents determined in oocytes expressing $Vssc1^{WT}/Vssc\beta$, $Vssc1^{MI}/Vssc\beta$, $Vssc1^{TI}/Vssc\beta$, $Vssc1^{LF}/Vssc\beta$ (A and D, respectively); $Vssc1^{MITI}/Vssc\beta$, $Vssc1^{MILF}/Vssc\beta$, $Vssc1^{TILF}/Vssc\beta$ (B and E, respectively); and $Vssc1^{MITILF}/Vssc\beta$ (C and F, respectively). Data points (means $\pm$ SD) were obtained from 3–6 separate oocytes.

Table 1
Sequence of oligonucleotide primes used for site-directed mutagenesis to insert point mutations into *Mut^{WT}*

Point mutation	Primer	Sequence
M827I	5fly-MI	5' CCATGGATCATCAGACATTAAATCCGGAATTAGAGAAGG 3'
	3fly-MI	5' CCTTCTCTAATCCGGATTAAATGTCGTGATGCCATGG 3'
	5fly-TI	5' GGTGCAATGGGTAATCTGATAATTTGTACTTTGCATTATC 3'
T929I	3fly-TI	5' GATAATGCCAAAGTACAAATATCAGATTACCAATGCACC 3'
L932F	5fly-LF	5' GGTAAATCTGACATTGTGATTTTGGCATTATCATCTTTCATC 3'
	3fly-LF	5' GATGAAGATGATAATGCAGAAATACAAATGTCAGATTACC 3'

Expression rates (%) and transient peak currents (I_{max}) of wild type and specifically mutated *Vssc1* co-expressed with *Vsscβ* in *Xenopus* oocytes

AA residue ^a		N ^b	Vssc1 variant	% Expression ^c	I _{max} ± SD (n) ^d
827	929				
M	T	84	Vssc1 ^{WT}	53.57	1.51 ± 0.85 (13)
I	T	46	Vssc1 ^{MI}	56.52	1.46 ± 1.37 (16)
M	I	58	Vssc1 ^{TI}	27.59 ^e	0.59 ± 0.17 (10) ^f
M	T	52	Vssc1 ^{LF}	32.73 ^e	2.32 ± 1.92 (11)
I	I	36	Vssc1 ^{MITI}	38.89	2.33 ± 1.35 (13)
I	T	50	Vssc1 ^{MILF}	58.00	1.46 ± 0.98 (15)
M	I	50	Vssc1 ^{TILF}	26.00 ^e	0.62 ± 0.16 (7) ^f
I	I	98	Vssc1 ^{MITILF}	47.96	0.84 ± 0.43 (10)

^a*Vssc1* variants are identified by the amino acid residues at positions 827, 929 and 932. The MTL (M827/T929/L932) variant is wildtype *Vssc1* (*Vssc1*^{WT}).

^bN, total number of oocytes co-injected with *Vssc1* and *Vsscβ* cRNAs.

^c% Expression = (Oocytes producing TTX-sensitive sodium currents/total oocytes co-injected with the same amount of *Vssc1* and *Vsscβ* cRNAs) × 100.

^dNumber of eggs used to measure transient peak current.

^eIndicates percent expression of the specifically mutated *Vssc1*/*Vsscβ* variant is significantly less than that of *Vssc1*^{WT}/*Vsscβ* (*t*-test, *P* < 0.05).

^fAmplitude of transient peak current significantly reduced compared to that of *Vssc1*^{WT}/*Vsscβ* (*t*-test, *P* < 0.05).

Voltage dependence of activation and steady-state inactivation of wildtype and specifically mutated Vssc1 co-expressed with Vsscβ in *Xenopus* oocytes

Table 3

Amino acid residue ^d			Vssc1 variant			Activation			Steady-state inactivation		
827	929	932				N	V _{0.5} ^b , mV ± SD	K ^c ± SD	N	V _{0.5} ^d , mV ± SD	K ^c ± SD
M	T	L	Vssc1 ^{WT}			10	-13.2 ± 3.0	4.7 ± 2.5	5	-33.8 ± 3.2	6.9 ± 2.8
I	T	L	Vssc1 ^{MI}			14	-14.9 ± 3.4	4.6 ± 2.8	5	-34.5 ± 2.9	6.3 ± 3.0
M	I	L	Vssc1 ^{TI}			5	-13.9 ± 2.1	4.6 ± 1.7	3	-28.8 ± 1.0	6.7 ± 0.9
M	T	F	Vssc1 ^{LF}			4	-16.7 ± 2.3	4.8 ± 1.9	6	-29.3 ± 2.6	5.3 ± 2.2
I	I	L	Vssc1 ^{MITI}			8	-17.2 ± 2.5 ^e	3.8 ± 2.1	4	-27.2 ± 1.7 ^e	4.9 ± 1.7
I	T	F	Vssc1 ^{MILF}			5	-15.6 ± 1.9	4.0 ± 1.5	4	-31.2 ± 2.4	5.2 ± 2.1
M	I	F	Vssc1 ^{TILF}			3	-12.5 ± 0.5	4.9 ± 0.4	3	-26.2 ± 3.5 ^e	6.7 ± 3.0
I	I	F	Vssc1 ^{MITILF}			7	-10.0 ± 1.2 ^e	4.9 ± 1.0	6	-25.0 ± 4.5 ^e	8.2 ± 3.9

^a Vssc1 channel variants are identified by the amino acid residues at positions 827, 929, and 932. The MTL (M827/T929/L932) variant is wildtype Vssc1 channel (Vssc1^{WT}).

^b Half maximal potential determined from Eq. 3.

^c Slope factor.

^d Half maximal potential determined from Eq. 4.

^e Significantly different from Vssc1^{WT}/Vsscβ channel (t-test, *p* < 0.05).

Table 4

Comparative permethrin (200 μ M) sensitivity reduction (PSR) in Vssc1 variants using mean values of normalized maximum tail current amplitude, normalized late current amplitude and tail current decay constant (τ)

Vssc1 variant	N	Maximum tail current		Late current		Tail current decay constant	
		Normalized mean ^d $\pm$ SD (CV ^b)	PSR (fold) ^c	Normalized mean ^d $\pm$ SD (CV ^b)	PSR (fold) ^c	Mean $\tau^e \pm$ SD, ms (CV ^b)	PSR (fold) ^c
Vssc1 ^{WT}	4	0.443 $\pm$ 0.158 (0.357)	-	0.224 $\pm$ 0.055 (0.246)	-	549.9 $\pm$ 21.4 (0.039)	-
Vssc1 ^{MI}	6	0.149 $\pm$ 0.094 (0.631)	3.0	0.089 $\pm$ 0.052 (0.584)	2.5	159.0 $\pm$ 28.8 (0.181)	3.5
Vssc1 ^{TI}	4	Ng ^f	-	Ng ^f	-	ND ^g	-
Vssc1 ^{LF}	5	0.149 $\pm$ 0.101 (0.678)	3.0	0.087 $\pm$ 0.060 (0.690)	2.6	135.4 $\pm$ 22.6 (0.167)	4.1
Vssc1 ^{MITI}	3	Ng ^f	-	Ng ^f	-	ND ^g	-
Vssc1 ^{MILF}	5	0.065 $\pm$ 0.047 (0.723)	6.8	0.062 $\pm$ 0.058 (0.935)	3.6	70.6 $\pm$ 8.6 (0.122)	7.8
Vssc1 ^{TILF}	3	Ng ^f	-	Ng ^f	-	ND ^g	-
Vssc1 ^{MITILF}	3	Ng ^f	-	Ng ^f	-	ND ^g	-

^aNormalized mean of maximum tail current = (tail current with 200 μ M permethrin - tail current determined prior to the permethrin treatment)/transient peak current determined prior to the permethrin treatment.

^bCoefficient of variation (CV) = standard deviation (SD)/mean (M).

^cPermethrin sensitivity reduction (PSR) = mean value of tail current, late current or tail current decay constant of Vssc1^{WT}/respective mean value of a mutant variant.

^dNormalized mean of late current = (late current with 200 μ M permethrin - late current determined prior to the permethrin treatment)/transient peak current prior to the permethrin treatment.

^eIn assays with 200 μ M permethrin, decay of the decreasing phase of tail currents from oocytes expressing sodium channel variants were fitted to a single exponential to determine the τ values.

^fNg indicates that the calculated mean value is negative. Since the estimated value is expected to be positive, it is conceivable that the mean value is close to zero and the resulting PSR should indicate that the mutant channel is completely insensitive to 200 μ M permethrin treatment.

^gND = not determined due to the fast deactivation ($\tau < 1$ ms). This causes the poor fit ($R^2 < 0.01$) of a single exponential and indicates that the mutant channel is completely insensitive to 200 μ M permethrin treatment.