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Novel sodium channel gene mutations in *Blattella germanica* reduce the sensitivity of expressed channels to deltamethrin

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Abstract

Pyrethroid insecticides alter the normal gating of voltage-gated sodium channels in the nervous system. Three sodium channel mutations (E434K, C764R, L993F) were recently identified in pyrethroid resistant German cockroach populations. In this report, we show that the L993F mutation decreased sodium channel sensitivity to the pyrethroid, deltamethrin, by five-fold in *Xenopus* oocytes. In contrast, neither E434K nor C764R alone decreased channel sensitivity to deltamethrin. However, E434K or C764R combined with L993F reduced deltamethrin sensitivity by 100-fold. Furthermore, concomitant presence of all three mutations (KRF) reduced channel sensitivity to deltamethrin by 500-fold. None of the mutations significantly affected channel gating. However, sodium current amplitudes from the mutant sodium channel carrying either E434K or C764R alone were much reduced compared to those of the wild-type channel or the channel carrying the double or triple mutations (KF, RF and KRF). These results indicated that evolution of sodium channel insensitivity in the German cockroach is achieved by sequential selection of a primary mutation L993F and two secondary mutations E434K and C764R, and concomitant presence of all three mutations dramatically reduced sodium channel sensitivity to deltamethrin.

Keywords

Knockdown resistance; Pyrethroids; Insecticide resistance; Sodium channel; *Xenopus* oocyte expression system

1. Introduction

The voltage-dependent sodium channel is a major target for a variety of neurotoxins including DDT and pyrethroid insecticides (Narahashi, 1986; Catterall, 1988; Hille, 1992; Zlotkin, 1999). These neurotoxins have well-defined effects on many functional properties

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of the sodium channel, such as conductance, ion selectivity, activation, and inactivation (Catterall, 1988). They are widely used as molecular probes for characterization of functional regions within sodium channel subunits (Catterall, 1992). Indeed, these neurotoxins are often classified according to their unique binding sites on the sodium channel (Catterall, 1988). For example, both DDT and pyrethroid insecticides, although structurally different, cause repetitive discharge or nerve membrane depolarization by modifying sodium channel gating (references in Narahashi, 1988; Soderlund and Bloomquist, 1989). Results from electrophysiological (such as Narahashi, 1988; Takeda and Narahashi, 1988; Song et al., 1996) and pharmacological studies (such as Jacques et al., 1980, Bloomquist and Soderlund, 1988; Brown et al., 1988; Lombet et al., 1988; Trainer et al., 1997) suggest a distinct receptor site(s) for pyrethroids. Although the electrophysiological effects of pyrethroids on sodium channels have been well-defined, our understanding of the molecular interactions between sodium channels and pyrethroids remains rudimentary. Because pyrethroids are highly lipophilic, pharmacological studies have been either impossible to perform or of limited utility due to very high levels of nonspecific ligand binding. Identification and electrophysiological characterization of mutations that affect sodium channel sensitivity to pyrethroids provide a powerful complementary approach, and should lead to a better understanding of sodium channel function and of the molecular interactions between sodium channels and pyrethroids that act on them.

Important sources of mutations that affect sodium channel interactions with DDT and pyrethroids are DDT- and pyrethroid-resistant insects. The intense use of these compounds has led to the selection of resistant populations in many insects. One important resistance mechanism is the called knockdown resistance (kdr) in housefly and kdr-type resistance in German cockroaches. It confers resistance to both knockdown (rapid paralysis) and killing by pyrethroids through reduced neuronal sensitivity to these compounds (references in Soderlund and Bloomquist, 1990). The kdr or kdr-type phenotype is associated with a leucine to phenylalanine mutation (L1014F in housflies, L993F in cockroaches) in IIS6 of the insect Para-homologous sodium channel protein in about a dozen insect species (Williamson et al., 1996; Miyazaki et al., 1996; Dong, 1997; Martinez-Torres et al. 1997, [1998]; Guerrero et al., 1997; Martinez-Torres et al., 1999a,b; Lee et al., 1999a). The super-kdr resistance phenotype in the house fly is associated with both the L1014F mutation in IIS6 and an M to T mutation (M918T) in the linker region between S4 and S5 of domain II (Williamson et al., 1996). In pyrethroid-resistant tobacco budworm (*Heliothis virescens*), the L1029H mutation in IIS6 (corresponding to L993F in cockroaches) or a V421M mutation in IS6 is associated with pyrethroid resistance (Park and Taylor, 1997; Park et al., 1997; Lee et al., 1999b).

Using the *Xenopus* oocyte expression system, Smith et al. (1997) showed that the house fly sodium channel carrying the L1014F mutation alone was 10-fold less sensitive to the pyrethroid cismethrin than the wild-type channel. The super-kdr M918T mutation, when combined with the L1014F mutation, further reduced sodium channel sensitivity to cismethrin (Lee et al., 1999c). Introduction of the two house fly mutations into the *Drosophila* Para protein greatly decreased channel sensitivity to deltamethrin (Vais et al., 2000). The V421M and L1029H sodium channel mutations associated with pyrethroid resistance in *Heliothis virescens* were also confirmed to reduce sodium channel sensitivity to the pyrethroid permethrin (Zhao et al., 2000). These studies clearly established that these sodium channel mutations play a significant role in sodium channel insensitivity to pyrethroids.

The L993F mutation was also identified in more than twenty pyrethroid-resistant German cockroach populations (Miyazaki et al., 1996; Dong, 1997; Dong et al., 1998). However, the

house fly super-kdr mutation was not found in any of the five highly resistant German cockroach populations (Dong et al., 1998). Instead, four novel *para* mutations (D58G, E434K, C764R and P1880L) were found to be associated with high-level pyrethroid resistance in this insect species (Liu et al., 2000). This raises the intriguing possibility that different *para*^{CSMA} gene mutations may have been selected in different insect species for high levels of pyrethroid resistance. In this study, we investigate the effects of E434K, C764R, and L993F mutations, which were present in all five highly resistant strains described by Liu et al. (2000), on cockroach Para^{CSMA} channel properties and sensitivity to a pyrethroid insecticide, deltamethrin.

2. Materials and methods

2.1. Polymerase chain reaction (PCR) amplification and cloning of a full-length para cDNA and D. melanogaster tipE cDNA

The nucleotide sequence of the coding region of the cockroach sodium channel gene *para*^{CSMA} was determined previously by sequencing overlapping cDNA clones (Dong, 1997). In this study, mRNA was isolated from the heads and thoraces of adult male German cockroaches of strain CSMA, which is susceptible to pyrethroids and DDT. The methods for RNA isolation and cDNA synthesis are the same as described in Dong (1997). For amplification of *para*^{CSMA} cDNA, the first strand cDNA was synthesized using a primer corresponding to the sequence in the 3′ untranslated region (5′-AAGAATGAGGCATCTCCGAG-3′). The entire coding region (ca. 6 kb) of *para*^{CSMA} was amplified by PCR using the eLONGase enzyme mix (Gibco/BRL, Rockville, MD). The amplification reaction mixture (50 μl) contained 0.5 μl cDNA, 0.2 μmol 5′ primer (5′-CCGGTACC*GCCACCATGG*CCGACGACTCGTCCTC AATCTCAGA-3′; Kozak sequence italics), 0.2 μmol 3′ primer (5′ -CCGGATCCAATCAAGCGAAGATGT GAGA-3′), 200 μM each dNTP, 1 U of eLONGase, 1.5 mM $MgCl_2$, and 1× PCR reaction buffer supplied by the manufacturer. To facilitate cloning, *Kpn*I and *Bam*H1 restriction site sequences were added to the 5′ and 3′ primers, respectively. The sequence of the 5′ primer was designed to conform to the Kozak sequence for high-efficiency translation. The T^{+4} to G^{+4} change results in the Ser to Ala substitution. The PCR amplification was performed for 30 cycles of 30 s at 94°C, 30 s at 58°C, and 7 min at 68°C followed by incubation at 68°C for 10 min. The amplified full-length cDNA with *Kpn*I and *Bam*H1 sites attached to the 5′ and 3′ ends, respectively, was cloned into pGH19 (kindly provided by Dr B. Ganetzky, University of Wisconsin, Madison). The entire insert of one clone, KD1, was sequenced. The *D. melanogaster tipE* cDNA was amplified and cloned into pBluescript KS(+) using PCR primers based on the published sequence (Feng et al., 1995).

2.2. Site-directed mutagenesis

KD1 was mutagenized to generate a series of mutant constructs with various combinations of the three mutations, E434K, C764R, and L993F. Specifically, a 2.3-kb *Nde*I fragment containing all three nucleotides to be mutated was excised from KD1 and cloned into pAlter 1 (Promega Corp., Madison, WI) for site-directed mutagenesis. Point mutations were introduced using the Altered Sites II in vitro Mutagensis System (Promega Corp.) and confirmed by DNA sequencing. The mutated *Nde*I fragments were excised from pAlter1 and cloned back into pGH19 containing the remainder of the KD1 sequence to produce the corresponding mutant constructs.

2.3. Expression of Para^{CSMA} sodium channels in Xenopus oocytes

Oocytes were obtained surgically from oocyte-positive female *Xenopus laevis* (Nasco, Ft. Atkinson. WI) and incubated with 1 mg/ml Type IA collagenase (Sigma Co., St. Louis, MO) in Ca^{2+}-free ND 96 medium, which contains 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5

mM HEPES, pH 7.5. Follicle cell remaining on the oocytes were removed with forceps. Isolated oocytes were incubated in ND-96 medium containing 1.8 mM $CaCl_2$ supplemented with 50 μg/ml gentamicin, 5 mM pyruvate, and 0.5 mM theophylline (Goldin, 1992). Healthy stage V–VI oocytes were used for cRNA injection. To prepare cRNA for oocyte injection, plasmid DNA of the *para* or *tipE* construct was linearized with *Not*I, which does not cut the insert, followed by in vitro transcription with T7 polymerase using the mMESSAGE mMACHINE kit (Ambion). $para^{CSMA}$ cRNA (1 ng) and *tipE* cRNA (1 ng) were co-injected into oocytes, which were incubated at 19°C for three–seven days.

2.4. Electrophysiological recording and analysis

Methods for electrophysiological recording and data analysis are similar to those described previously (Kontis and Goldin, 1993). Sodium currents were recorded using standard two-electrode voltage clamping. The borosilicate glass electrodes were filled with filtered 3 M KCl and had resistance less than 0.5 MΩ. Currents were measured using the oocyte clamp instrument OC725C (Warner Instrument Corp. Hamden, CT), Digidata 1200A interface (Axon Instrument, Foster City, CA) and pCLAMP 6 software (Axon Instrument Foster City, CA). All experiments were performed at room temperature (20–22°C). Capacitive transient and linear leak currents were corrected using P/N subtraction technique or by subtraction of records obtained in the presence of 20 nM tetrodotoxin (TTX), which completely blocks Para sodium channels.

For application of deltamethrin, the disposable perfusion system developed by Tatebayashi and Narahashi (1994) was used. Briefly, the test solution was transferred into a Petri dish placed on a supportstand. Two glass capillary tubes (10 cm in length) connected together with a short length of Tygan tubing were used to aid solution flow from the Petri dish to the recording chamber. The solution flow was controlled by hydro-static force created by adjusting the level of the Petri dish relative to the recording chamber. Disposable recording chambers (1–1.5 ml volume) were made with glue dams on glass slides. Because deltamethrin is extremely lipophilic, recording chambers, perfusion system, and the glass agarose bridges connecting the oocyte chamber with the ground electrode chamber were all discarded after a single use. The deltamethrin stock solution (100 mM) was prepared in dimethylsulfoxide (DMSO). The working solutions were made in ND-96 medium immediately before use. Effects of deltamethrin on sodium channel tail currents reached a steady-state level within 5 min after perfusion.

The voltage-dependence of sodium channel conductance (G) was calculated by measuring the peak current at test potentials ranging from −120 to +60 mV in 5 mV increments and dividing by (V–V_{rev}), where V is the test potential and V_{rev} is the reversal potential for sodium. Reversal potentials were determined from the I–V curves. Peak conductance values were fit with a two-state Boltzmann equation of the form $G=1-[1+\exp(V-V_{1/2})/k]^{-1}$, in which V is the potential of the voltage pulse, $V_{1/2}$ is the half-maximal voltage for activation, and k is the slope factor.

The voltage-dependence of sodium channel inactivation was determined using 200 ms inactivating pre-pulses from a holding potential of −120 to +40 mV in 5 mV increments, followed by test pulses to −5 mV for 12 ms. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude, and plotted as a function of the pre-pulse potential. The data were fit with a two-state Boltzmann equation of the form $I=I_{max}*[1+(\exp(V-V_{1/2})/k)]^{-1}$, where I_{max} is the maximal current evoked, V is the potential of the voltage pulse, $V_{1/2}$ is the voltage at which 50% of the current is inactivated (the midpoint of the inactivation curve), and k is the slope factor.

The percentage of channels modified by deltamethrin was calculated using the equation $M=[I_{tail}/(E_h - E_{Na})]/[I_{Na}/(E_t - E_{Na})]\times 100$ (Tatebayashi and Narahashi, 1994), in which I_{tail} is the maximal tail current amplitude, E_h is the potential to which the membrane is repolarized, E_{Na} is the reversal potential for sodium current determined from the *I–V* curve, I_{Na} is the amplitude of the peak current during depolarization before deltamethrin exposure, and E_t is the potential of step depolarization. The concentration–response data were fitted to the Hill equation: $M=M_{max}/1+(EC_{50}/[\text{deltamethrin}])^n$, where [deltamethrin] and EC50 represent the concentration of deltamethrin and the concentration to produce the half-maximal effect, respectively, *n* represents the Hill coefficient, and the M_{max} is the maximal percentage of sodium channels modified.

3. Results

3.1. Functional expression of Para^{CSMA} in Xenopus oocytes

We isolated a full-length cockroach *para*^{CSMA} cDNA clone, KD1, by RT-PCR. Four amino acid sequence discrepancies, R502G, L1285P, V1685A and I1806L were found between KD1 and the previously published sequence (Dong, 1997). KD1 contains alternative exon 1 but not alternative exon 2 and another alternative exon (^{727}VSIYYFPT^{735}, Dong, 1997). Expression of KD1 in *Xenopus* oocytes confirmed that KD1 encodes a functional sodium channel protein. Specifically, sodium currents with a peak current of approximately 2–5 μA were detected on day 4 or 5 in most oocytes co-injected with *para*^{CSMA} and *tipE* cRNA (1 ng each). The sodium currents were completely inhibited by 10 nM TTX (Fig. 1B). Sodium current traces from a representative oocyte are shown in Fig. 1A. The oocyte was clamped at a holding potential of −120 mV and depolarized to test potentials between −80 and 60 mV in 5 mV increments. Sodium current through the cockroach Para^{CSMA} channel was detected at approximately −40 mV and reached peak values at −10 mV (Fig. 1C). The average relative sodium conductance was calculated as described in Section 2 and plotted as a function of depolarizing test potentials (Fig. 1D). The voltage for half-maximal activation was −29.0±1.3 mV and the slope factor was 5.5±0.5 (Table 1). To determine the voltage-dependence of steady-state inactivation, oocytes were held at −120 mV and depolarized with a series of 200-ms inactivating prepulses from −120 to +40 mV in 5 mV increments, followed by 12-ms test pulses to −5mV to measure channel availability. The voltage-dependence of steady-state inactivation was obtained by plotting the normalized peak current as a function of prepulse potentials, as described in Section 2 (Fig. 1D). The voltage for half-maximal inactivation was −42.4±0.4 mV and the slope factor was 5.0±0.1 (Table 1).

3.2. Effects of kdr-type mutations on channel expression and gating properties

Five *para*^{CSMA} mutations (D58G, E434K, C764R, L993F, and P1880L) were found to be associated with kdr-type resistance in the German cockroach (Liu et al., 2000). The L993F mutation was detected in twenty of twenty four recently field-collected pyrethroid-resistant populations (Dong, 1997; Dong et al., 1998). The E434K and C764R mutations were found to coexist with the L993F mutation in only the five most highly pyrethroid-resistant populations (Liu et al., 2000). The D58G and P1880L mutations were found in only one of the five populations (Liu et al., 2000). We hypothesized that the L993F mutation is responsible for a low level of knockdown resistance in cockroaches, whereas the other four mutations are responsible for high levels of kdr-type resistance. To test this hypothesis, we generated constructs with all eight possible combinations of the three most common mutations, E434K, C764R and L993F (Fig. 2). For simplicity, the mutants will be referred to by the single letter code for the new amino acid that is present, with multiple letters indicating the presence of multiple mutations, as listed in Table 1. We first examined the effects of the various combinations of mutations on the peak current of the sodium channel in oocytes. Mutant channels, Para^{CSMA-F}, Para$^{CSMA-KR}$, Para$^{CSMA-RF}$, Para$^{CSMA-KF}$ and

Para$^{CSMA-KRF}$ all expressed sodium currents with peak currents comparable to that of the wild-type channel (Table 2). The peak currents of mutant channels, Para^{CSMA-K} and Para^{CSMA-R}, however, were significantly (four- to five-fold) lower than that of the wild-type channel (Table 2).

Effects of mutations on voltage-dependence of activation and steady-state inactivation were also examined. Sodium currents recorded in oocytes expressing mutant channels exhibited voltage-dependence of activation and inactivation similar to those of the wild-type channel (Table 1), demonstrating that the gating properties of the mutant channels are apparently normal.

3.3. kdr-type Mutations altered the Para^{CSMA} channel sensitivity to deltamethrin

Pyrethroids alter sodium channel function by slowing inactivation and deactivation (Narahashi, 1988). Specifically, the sodium current during depolarization is prolonged, and the tail current associated with repolarization is greatly augmented. The pyrethroid-induced tail current has been used to quantify pyrethroid modification of the sodium channel (Lund and Narahashi, 1982; Vijverberg et al., 1982; Song et al., 1996; Lee et al., 1999c; Vais et al., 2000). Cohen and colleagues (Vais et al., 2000) compared two tail current recording protocols, one with a 500 ms depolarization from −100 to 0 mV and the other with a 100-pulse train of 5 ms depolarization from −100 to 0 mV. They found that the train of pulses produced a more pronounced tail current, suggesting that deltamethrin interacts with the open state of the channel (Vais et al., 2000). Therefore, for measurement of pyrethroid-induced tail current in this study, we adopted the recording protocol of a 100-pulse train of 5 ms depolarization from −120 to 0 mV with a 2-ms interval between each depolarization. Traces of tail current were recorded 5 min after application of each deltamethrin concentration. The deltamethrin-induced tail current increased initially and then gradually decreased, generating a hooked tail current (Fig. 3).

The minimum effective deltamethrin concentration required to elicit a detectable tail current is approximately 0.01 μM for the Para^{CSMA}, Para^{CSMA-K} and Para^{CSMA-R} channels (Fig. 3A–C). A large tail current was elicited at 1.0 μM (Fig. 3A–C). Concentrations above 1.0 μM resulted in large leakage currents that made it impossible to clamp the oocytes. Conversely, much higher concentrations of deltamethrin (10–100 μM) were required to elicit significant tail currents for Para^{CSMA-F}, Para$^{CSMA-KF}$, Para$^{CSMA-RF}$ and particularly Para$^{CSMA-KRF}$ channels (Fig. 3D, F–H), clearly demonstrating the importance of these mutations in Para^{CSMA} channel insensitivity to deltamethrin in the German cockroach. Surprisingly the Para$^{CSMA-KR}$ channel, which carries the two novel mutations E434K and C764R but not the L993F mutation, was more sensitive to deltamethrin than the wild-type channel or channels carrying only the E434K or C764R single mutation (Fig. 3E).

To quantify the differences in sensitivity to delta-methrin among channels, we calculated the percentage of channels modified by deltamethrin as described in Section 2 (Tatebayashi and Narahashi, 1994). The percentage modification was plotted as a function of the deltamethrin concentration (Fig. 4). Modification of 20% of sodium channels required five-fold more deltamethrin for the Para^{CSMA-F} channel, 100-fold more for the Para$^{CSMA-KF}$ and Para$^{CSMA-RF}$ channels, and 500-fold more for the Para$^{CSMA-KRF}$ channel, compared to the wild-type channel. The sensitivity of the Para^{CSMA-R} channel to deltamethrin was similar to that of the wild-type channel. Para^{CSMA-K} and Para$^{CSMA-KR}$ channels were two-fold and 10-fold, respectively, more sensitive to deltamethrin than the wild-type channel (Fig. 4).

The tail currents of Para$^{CSMA-KF}$, Para$^{CSMA-RF}$ and Para$^{CSMA-KRF}$ channels clearly decayed more completely than the tail currents of the other channels including the Para^{CSMA-F} channel (Fig. 3). To quantify this difference, the largest tail current in Fig. 3 was used for the

analysis of tail current decay for each channel. The tail current decay was best fitted with a single exponential equation. The time constants for $Para^{CSMA-KF}$, $Para^{CSMA-RF}$ and $Para^{CSMA-KRF}$ channels were significantly smaller than those of the other channels (Table 3).

4. Discussion

This is the first functional characterization of a cockroach sodium channel $Para^{CSMA}$ expressed in *Xenopus* oocytes. Similar to the *D. melanogaster* Para and house fly Vssc1 sodium channels, the cockroach $Para^{CSMA}$ sodium channel is TTX-sensitive (an EC_{50} of 0.9 nM) and requires *Drosophila tipE*, a potential subunit of insect sodium channels (Feng et al., 1995; Warmke et al., 1997), for robust expression. Most importantly, we demonstrated a critical role of two novel *para*^{CSMA} gene mutations (E434K and C764R) in reduced $Para^{CSMA}$ channel sensitivity to deltamethrin. Specifically, we found that: (i) the previously characterized L993F mutation alone conferred only a low level of insensitivity to deltamethrin; (ii) E434K or C764R alone significantly reduced sodium peak currents but had no effect on $Para^{CSMA}$ channel sensitivity to deltamethrin; and (iii) when E434K and C764R were combined with L993F (i.e. $Para^{CSMA-KF}$, $Para^{CSMA-RF}$ and $Para^{CSMA-KRF}$ channels), however, sodium current magnitudes were restored to the wild-type level and channel sensitivity to deltamethrin was markedly reduced. These results confirm our previous speculation (Liu et al., 2000) that the E434K and C764R mutations, in the presence of L993F, are responsible for high levels of pyrethroid-resistance in the German cockroach.

In the house fly, the super-kdr mutation (M918T) in the linker connecting IIS4 and IIS5, when combined with the L to F mutation (L1014F in house fly), confers higher-level resistance to pyrethroids (Lee et al., 1999c). This super-kdr mutation is also present in pyrethroid-resistant horn flies (Guerrero et al., 1997). The fact that highly pyrethroid-resistant cockroaches contain the E434K or C764R mutations instead of the M918T mutation is somewhat unexpected. This variation in the mutations that cause higher levels of resistance is in contrast to the absolute conservation of the kdr-associated mutation (L1014F in house fly, and L993F in German cockroach in IIS6) in all characterized kdr and kdr-type insects (Williamson et al., 1996; Miyazaki et al., 1996; Dong, 1997; Guerrero et al., 1997; Martinez-Torres et al. 1997, 1998; Martinez-Torres et al., 1999a,b; Lee et al., 1999a). Therefore, these results establish that different sodium channel mutations, when combined with the L1014F mutation in house fly or the L993F mutation in German cockroach, can result in high-level resistance to pyrethroids. It remains an interesting question why distinct secondary mutations are selected in house fly and cockroach to enhance the level of pyrethroid resistance.

It is important to note that although L993F alone reduced channel sensitivity to a pyrethroid, as was observed for the house fly kdr mutation (L1014F) and the *Heliothis virescens* L1029H mutation, neither the E434K mutation nor the C764R mutation alone reduced $Para^{CSMA}$ channel sensitivity to pyrethroids. This is in sharp contrast to the house fly M918T mutation, which alone reduces sodium channel sensitivity to pyrethroids (Lee et al., 1999c). In fact, the $Para^{CSMA}$ channel bearing the E434K and C764R double mutation is more sensitive to deltamethrin (Fig. 3). Furthermore, we did not detect significant alteration of channel gating properties by any of the three single mutations or various mutation combinations. The housefly L1014F mutation, however, causes a depolarizing shift (ca. 6-mV) of voltage-dependence of activation (Lee et al., 1999c). A similar shift was observed when the *Heliothis virescens* L1029H mutation was introduced into the *Drosophila* Para channel (Zhao et al., 2000). However, when the housefly L1014F mutation was introduced into the *Drosophila* Para channel, no significant shift was observed (Vais et al., 2000). Our results are consistent with the latter.

The hooked tail currents induced by deltamethrin in this study have been observed in mammalian neurons and sodium channels expressed in oocytes (such as Lund and Narahashi, 1982; Vijverberg et al., 1982; Song et al., 1996; Vais et al., 2000). The initial increasing phase of the tail current is the result of the delayed closing of the inactivation gate upon termination of depolarization. The subsequent decay of the tail current reflects the slow rate of deactivation of the modified channels. The mutation combinations, RF, KF and KRF, not only reduce the channel sensitivity to deltamethrin, also cause more complete decay of the tail current possibly by increasing the rate of deactivation of the modified channels. An acceleration of tail current decay was previously observed for the house fly kdr and super-kdr mutations and the *Heliothis virescens* L1029H mutation (Smith et al., 1997; Vais et al., 2000; Zhao et al., 2000). However, the cockroach L993F mutation reduces the channel sensitivity to deltamethrin without acceleration of the decay of the tail current (Table 3 and Fig. 3).

An intriguing observation from this study is that the E434K or C764R mutation alone significantly reduced the peak sodium current by more than four-fold, whereas the L993F mutation alone did not affect the peak sodium current or channel gating (Tables 1 and 3). Reduction of peak sodium currents was also observed for house fly sodium channels carrying the M918T mutation (Lee et al., 1999c). The neutral effects of the L993F mutation alone on sodium channel peak current and gating can explain the genetic stability and presence of this mutation in all characterized kdr and kdr-type insect pest species/populations collected from diverse regions. However, the negative effect of the E434K or C764R mutation on peak sodium current indicates that insects carrying either of these two mutations may have decreased fitness. In support of this speculation, we have not detected any cockroaches containing only the E434K or C764R mutation (Liu et al., 2000; Z. Liu and K. Dong, unpublished data). The mechanism by which the E434K and C764R mutations decrease sodium current amplitudes is not known. It is possible that these substitutions alter the folding or assembly of the $Para^{CSMA}$ protein, which would result in fewer functional channels, or they might decrease either the single channel conductance or probability of channel opening, or they decrease the interaction of $Para^{CSMA}$ with TipE.

Interestingly, when E434K or C764R is combined with L993F, normal peak current amplitudes were observed (Table 3). Similarly, the concomitant expression of the L1014F and M918T mutations in the house fly sodium channel results in normal peak current amplitudes (Lee et al., 1999c). Based on this observation, we speculate that an initial selection of the L993F mutation (most likely by DDT; see Dong, 1997) must have provided a platform for subsequent selection of the E434K and C764R mutations by pyrethroids in the German cockroach. Presence of the L993F mutation in the $para^{CSMA}$ gene would compensate for the functional constraint imposed by E434K and C764R mutations. Therefore, the L993F mutation must have played a crucial role in the evolution of high-level pyrethroid resistance in cockroaches, allowing selection of additional $para^{CSMA}$ gene mutations that otherwise would decrease the function of the $Para^{CSMA}$ channel. Furthermore, we found that the $Para^{CSMA\text{-}KR}$ channel (double mutations in the absence of L993F) was more susceptible to deltamethrin (Fig. 3) and would unlikely have survived pyrethroid selection in the field. All these observations suggest a ‘successive mutation’ mechanism by which German cockroaches adapted to pyrethroid selective pressure. A similar mechanism has been proposed by Lee et al. (1999c) to explain the selection of the super-kdr mutation (M918T) in the housefly. Such a mechanism is in contrast to the ‘mutation recombination’ mechanism, in which preexisting intragenic resistance mutations in the acetylcholinesterase gene are recombined to produce higher levels of resistance to organophosphates (Mutero et al., 1994). Thus, the successive mutation mechanism appears to represent an alternative paradigm for evolution of insecticide resistance.

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Abbreviations

DDT	1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane
kdr	knockdown resistance
TTX	tetrodotoxin

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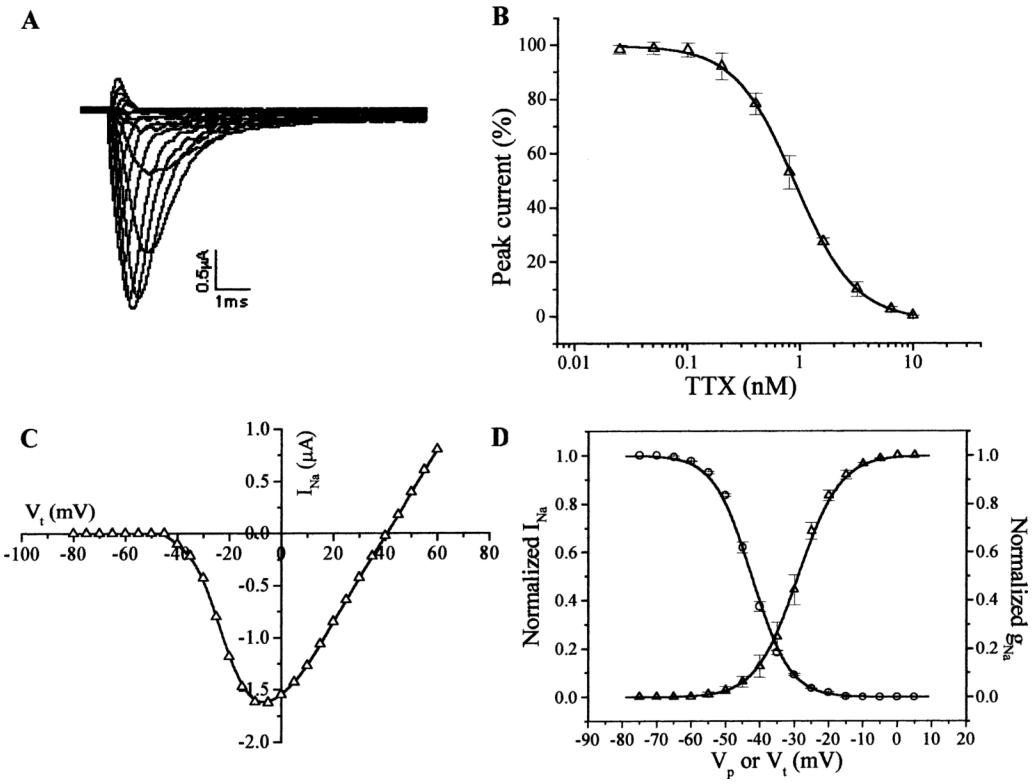

Fig. 1.

Functional expression of a cockroach Para^{CSMA} sodium channel in *Xenopus* oocytes (coexpressed with *D. melanogaster tipE*). (A) Sodium current traces. Sodium currents were recorded from a holding potential of −120 mV during depolarizations from −80 to 65 mV in 5 mV increments. (B) Dose-dependent block of sodium current by TTX. Amplitudes of the peak currents measured in the presence of various concentrations of TTX were normalized using the amplitude of the maximal peak current in the absence of TTX. The data were fitted with a sigmoid curve; EC_{50} is 0.9 nM. Symbols represent means and error bars indicate the SEM for five oocytes. (C) Sodium current–voltage relation (*I–V* curve). Current were recorded as described for (A), and the peak current amplitude is plotted versus the depolarization potential. Symbols represent means and error bars indicate the SEM for five oocytes. (D) Normalized conductance–voltage (*G–V*) and inactivation curves. The peak current in (C) was converted to conductance as described in Section 2 and plotted against the depolarizing voltage (triangles). The voltage-dependence of inactivation was determined using 200 ms inactivating pre-pulses from a holding potential of −120 to +40 mV in 5 mV increments, followed by test pulses to −5 mV for 12 ms. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude, and plotted as a function of the pre-pulse potential (circles). The smooth curves represent the best fit using Boltzmann equations, as described in Section 2. Symbols represent means; error bars indicate the SEM from three oocytes.

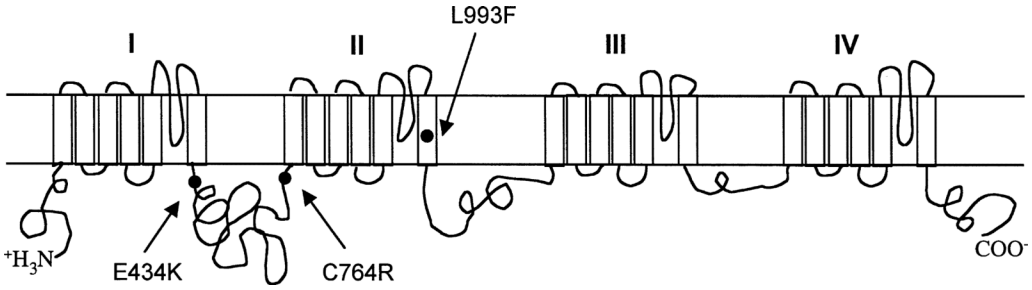

Fig. 2.

Schematic drawing of the cockroach Para^{CSMA} sodium channel protein indicating kdr-associated mutations. Four homologous domains (I–IV) and six transmembrane segments (S1–6) are shown. The three kdr-associated mutations (E434K, C764R, L993F) used in this study are indicated.

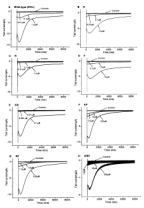

Fig. 3.

Para^{CSMA} channel sensitivity to deltamethrin. (A)–(H) Tail currents induced by deltamethrin in oocytes expressing the wild-type and mutant Para^{CSMA} channels. Tail currents were recorded in response to a 100-pulse train of 5 ms depolarizations from −120 to 0 mV with a 2-ms interval between each depolarization. K, R, F, KR, KF, RF, KRF represent sodium channels carrying E434K, C764R, L993F, E434K+C764R, E434K+L993F, C764R+L993F and E434K+C764R+L993F, respectively. Note the differences in the scales of tail current and also the concentrations of deltamethrin. At 100 μM, the recording solution showed a slight cloudiness, indicating that deltamethrin was not completely soluble at this concentration.

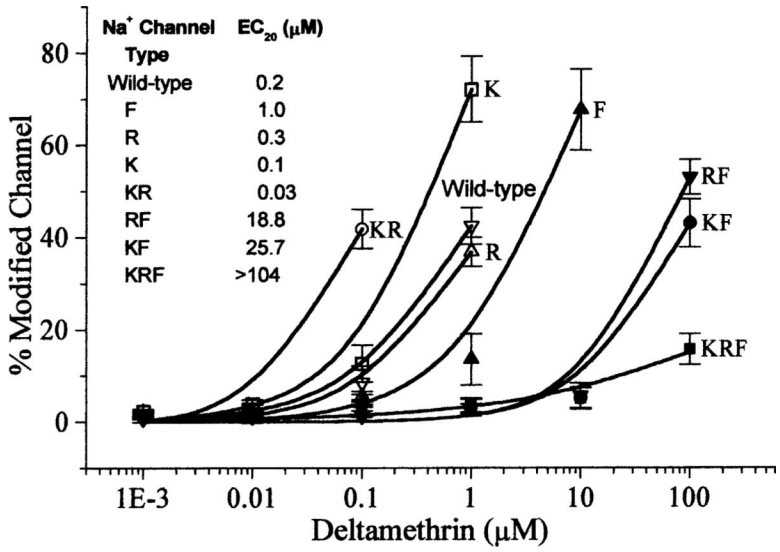

Fig. 4.

Quantification of Para^{CSMA} and mutant channel sensitivity to deltamethrin. The percentage of channels modified by deltamethrin was determined using the equation $M=[I_{tail}/(E_h–E_{Na})]/[I_{Na}/(E_t–E_{Na})] \times 100$ (Tatebayashi and Narahashi, 1994) (see Section 2). Percentage of modification of sodium channels is plotted as a function of delta-methrin concentration. The data were fitted with the Hill equation (see Section 2). EC_{20} values (inset) are derived from the fitted curves. Hill coefficient ranges 0.8–1.3 for different channels, except for the KRF channel, which gives a Hill coefficient of 0.4. Each point represents mean ± SEM (pooled data from five oocytes).

Table 1

Voltage dependence of activation and inactivation of Para^{CSMA} sodium channels

Na^+ channel type	Mutations	Activation[a]		Inactivation[a]	
		$V_{1/2}$ (mV)	k (mV)	$V_{1/2}$ (mV)	k (mV)
Para^{CSMA}	Wild-type	–29.0±1.3	5.5±0.5	–42.4±0.4	5.0±0.1
Para^{CSMA-F}	L993F	–27.8±2.4	6.2±0.4	–44.0±1.9	5.3±0.4
Para^{CSMA-R}	C764R	–27.9±4.5	5.6±0.6	–43.8±1.2	5.6±0.3
Para^{CSMA-K}	E434K	–25.7±3.7	5.4±1.0	–43.2±0.9	5.4±0.3
Para$^{CSMA-KF}$	E434K, L993F	–30.3±4.1	5.1±0.7	–39.3±1.1	5.1±0.8
Para$^{CSMA-RF}$	C764R, L993F	–24.2±2.7	5.3±0.7	–40.6±0.4	5.1±0.3
Para$^{CSMA-KR}$	E434K, C764R	–24.5±4.8	5.7±0.6	–44.4±3.2	5.6±0.2
Para$^{CSMA-KRF}$	E434K, C764R, L993F	–27.9±8.3	5.9±0.8	–40.9±1.7	5.2±0.8

[a]The voltage-dependence of conductance and inactivation data were fit with two-state Boltzmann equations, as described in Section 2, to determine $V_{1/2}$, the voltage for half-maximal conductance or inactivation and k, the slope factor for conductance or inactivation. Each value represents the mean ± SEM for three oocytes.

Table 2

Peak current amplitudes of wild-type and mutant Para^{CSMA} sodium channels in *Xenopus* oocytes

Na^+ channel type	I_{max} (μA)[a]	N[b]
Para^{CSMA}	5.59±1.20	12
Para^{CSMA-F}	3.89±1.44	6
Para^{CSMA-R}	0.96±0.24	4
Para^{CSMA-K}	1.29±0.23	5
Para$^{CSMA-RF}$	5.77±1.02	14
Para$^{CSMA-KR}$	5.90±1.17	9
Para$^{CSMA-KF}$	5.98±0.83	15
Para$^{CSMA-KRF}$	7.04±1.26	6

[a] Amplitude of the maximal peak current measured during a 20 ms depolarization from –120 to –10 mV.

[b] Number of oocytes.

Table 3

Time constant of tail current decay induced by deltamethrin

Na^+ channel type	τ^a (ms)	N^b
$Para^{CSMA}$	1537±737	20
$Para^{CSMA\text{-}F}$	1408±353	12
$Para^{CSMA\text{-}R}$	1518±227	7
$Para^{CSMA\text{-}K}$	1679±328	7
$Para^{CSMA\text{-}RF}$	1102±77[c]	11
$Para^{CSMA\text{-}KR}$	1677±357	14
$Para^{CSMA\text{-}KF}$	1034±96[c]	7
$Para^{CSMA\text{-}KRF}$	1042±130[c]	9

[a] The largest deltamethrin-induced tail currents (see Fig. 3) was fitted with a single exponential equation, as described in Section 2. Each value represents the mean ± SEM.

[b] Number of oocytes.

[c] Indicates a statistically significant difference compared to the wild-type channel at $p<0.05$.