Published in final edited form as:

Insect Biochem Mol Biol. 2000 October ; 30(10): 991–997.

Novel point mutations in the German cockroach *para* sodium channel gene are associated with knockdown resistance (*kdr*) to pyrethroid insecticides

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Abstract

Knockdown resistance (*kdr*) to pyrethroid insecticides has been attributed to point mutations in the *para* sodium channel gene in more than a half dozen insect pest species. In this study, we identified two novel *para* mutations in five highly resistant *kdr*-type German cockroach strains. The two mutations, from glutamic acid (E434) to lysine (K434) and from cysteine (C764) to arginine (R764), respectively, are located in the first intracellular linker connecting domains I and II. E434K is located near the beginning of the linker (closest to domain I), whereas C764R is found toward the end of the linker (closest to domain II). Two additional mutations from aspartic acid (D58) to glycine (G58), and from proline (P1880) to leucine (L1888), respectively, were found in one of the resistant strains. The four mutations coexist with the previously identified leucine to phenylalanine (L993F) *kdr* mutation in IIS6, and are present only in the highly resistant individuals of a given strain. These findings suggest that these mutations might be responsible for high levels of knockdown resistance toward pyrethroid insecticides in the German cockroach.

Keywords

Ion channel; Insecticide resistance

1. Introduction

For several decades, pyrethroid insecticides have been used widely to control many insect pests. Because of the intensive use of pyrethroids, however, many pest populations have developed resistance to these compounds. One class of the most important resistance mechanisms recognized is knockdown resistance (*kdr*). It confers resistance to both knockdown (rapid paralysis) and killing by pyrethroids and dichloro diphenyl trichloroethane (DDT) through reduced neuronal sensitivity to these compounds (see Soderlund and Bloomquist, 1990 and references therein).

The primary target site for pyrethroids is voltage-dependent sodium channels in the nervous system (Narahashi, 1988). An insect sodium channel gene, *para*, was first identified in *Drosophila* (Loughney et al., 1989). Orthologs for *para* have been isolated from several

other insect species, including the German cockroach. The deduced amino acid sequence of Para shares striking homology with all known sodium channel α -subunits in overall organization. Each consists of four repeated homologous domains (I–IV), having six membrane-spanning segments (S1–6) in each domain. Expression of Para in *Xenopus* oocytes confirmed that *para* encodes a functional voltage-gated sodium channel (Feng et al., 1995; Warmke et al., 1997).

Recent studies show that point mutations in the Para sodium channel protein are responsible for *kdr* and super-*kdr* (which confers much higher levels of resistance than *kdr*) resistance in insects. The *kdr* resistance in the house fly and German cockroach is associated with a leucine (L) to phenylalanine (F) mutation in segment 6 of domain II (IIS6) (Williamson et al., 1996; Miyazaki et al., 1996; Dong, 1997). The super-*kdr* resistance in house fly is associated with an additional methionine (M) to threonine (T) mutation in the linker region between S4 and S5 of domain II (Williamson et al., 1996). The house fly sodium channel carrying these two mutations is much less sensitive to the pyrethroid cismethrin than the wild-type when expressed in *Xenopus* oocytes (Smith et al., 1997; Lee et al., 1999). The L to F *kdr* mutation also has been detected in other pyrethroid resistant insects, including horn flies (*Haematobia irritans*) (Jamroz et al., 1998), mosquitoes (*Anopheles gambiae*) (Martinez-Torres et al., 1998) and aphids (*Myzus persicae*) (Martinez-Torres et al., 1999). The L to H (histidine) mutation at the same position is found in pyrethroid-resistant *Heliothis virescens* (Park and Taylor, 1997). One additional mutation (from valine to methionine in IS6) is associated with pyrethroid resistance in several *Heliothis virescens* strains (Park et al., 1997; Lee et al., 1999). The second mutation (M to T) in super-*kdr* house flies also was detected in horn fly (Jamroz et al., 1998).

In a recent survey, we found the L to F *kdr* mutation (IIS6) in 20 of 24 field-collected pyrethroid-resistant German cockroach strains (Dong et al., 1998). However, the M to T mutation detected in super-*kdr* house flies was absent in all five of the highly resistant cockroach strains (Dong et al., 1998). In the present study, we conducted further sequence analysis of cockroach *para* from five highly resistant German cockroach strains that possess the L to F mutation in IIS6. We have identified four additional mutations associated with the high level of *kdr* in the German cockroach.

2. Materials and methods

2.1. Strains

Two susceptible strains, CSMA and Orlando (Koehler and Patterson, 1986), Ectiban-R, a *kdr* strain (Scott et al., 1990), and eight recently field-collected pyrethroid-resistant German cockroach strains were used in this study. Of the eight recently field-collected pyrethroid-resistant German cockroach strains, five (Malo, Pinellis 214, Marietta, Swine and Fuerte) had been used in previous studies and were known to possess the L993F mutation (Dong et al., 1998), and three (Aves, Pinellis 417 and NASJAX) were collected from the field and characterized more recently (Valles, 1998; Valles et al., 2000).

2.2. Bioassays

The field-collected pyrethroid-resistant German cockroach strains were heterogeneous for *kdr*, so bioassays using a pyrethroid, cypermethrin, were conducted to separate knockdown susceptible from resistant individuals (Dong et al., 1998). Separation was achieved by topically treating 50 adult male cockroaches with piperonyl butoxide (PBO) and *S,S,S*-tributyl phosphorotrithioate (DEF) (100 and 30 μ g/cockroach, respectively) to minimize the contribution of detoxification. One hour later, two groups of 25 treated cockroaches were each placed into a glass Mason jar (1 pint) coated with cypermethrin at a concentration of

300 µg/jar. With this dose, individuals were all knocked down during a 4 h bioassay. The first five and last five knocked-down individuals were collected for isolating total RNA for subsequent polymerase chain reactions (PCR)/sequencing analyses.

2.3. RNA isolation and cDNA synthesis

Total RNA and poly(A⁺) RNA were isolated from cockroach heads and thoraces, using RNA isolation kits (Promega) according to the manufacturer's instructions. First strand cDNA was synthesized from 5 µg of total RNA using SuperScript II RNase H⁻ reverse transcriptase (GIBCO/BRL). A *para*-specific antisense primer (no. 18 in Table 1), complementary to the sequence at the 3' end of the *para* coding sequence, was used in cDNA synthesis. To increase the specificity of cDNA, the temperature for reverse transcription reaction was raised to 50°C instead of 42°C as recommended in the product manual.

2.4. PCR, cloning and sequencing

The first strand cDNA was used as template to amplify *para* cDNA. PCRs were performed in a Perkin-Elmer 480 thermal cycler using Taq polymerase (Gibco/BRL). PCR mix (50 µl) contained: 1 µl cDNA, 5 µl 10x PCR buffer, 0.5 µM of each primer, 200 µM of each dNTP, 1.5 mM MgCl₂ and 2.5 U Taq polymerase. PCR was started by addition of polymerase at high temperature (94°C) prior to cycling. The PCR conditions were: 30 cycles of 30 s at 94°C, 30 s at 58°C, and 1 min at 72°C. The PCR products were extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) followed by agarose gel electrophoresis. The Prep-A-Gene kit (Bio-Rad) was used to isolate the PCR products from agarose gel for cloning or direct sequencing. Sequence was determined in the W.M. Keck Laboratory at Yale University.

Direct sequencing of the PCR products generated decent sequence results, except for certain regions where alternative splice sites reside. For sequence analysis of these regions, we cloned the PCR products and sequencing inserts from multiple clones.

3. Results and discussion

Compared with the strain Ectiban-R, which contains only the L to F *kdr* mutation, the five strains (Malo, Pinellis 214, Fuerte, Pinellis 417 and NASJAX) were significantly more resistant to knockdown by cypermethrin (Table 2). Our previous study (Dong et al., 1998) showed that the *kdr* individuals in all of these strains possessed the L993F mutation. However, they all lack the second M to T mutation, which is associated with super-*kdr* in the house fly. We hypothesized that additional mutations in the *para* gene may be responsible for the higher level of *kdr* resistance in these cockroach strains. Our hypothesis was not without precedent. Mutero et al. (1994) reported that five point mutations in the acetylcholinesterase gene (*Ace*) of *Drosophila* were responsible for resistance to various organophosphorus and carbamate insecticides. They showed that a combination of several point mutations within the *Ace* gene conferred greater levels of resistance than any single point mutation.

To identify possible additional mutations, we amplified *para* cDNA fragments encoding the four transmembrane domains using RNA isolated from the last five knocked-down individuals of Malo, Pinellis 214, Fuerte, Pinellis 417 and NASJAX strains. Equal amount of PCR products from five individual cockroaches were pooled before sequencing. The primers used in PCR and sequencing are listed in Table 1. As reported previously, our method was capable of detecting the L993F mutation if it was present in at least one of five pooled PCR reactions from five individuals (Dong et al., 1998). In this study, we employed

the same detection method to determine whether additional *para* mutations were present in these strains. We first confirmed the previous finding that the last five individuals of all five strains, Malo, Pinellis 214, Pinellis 417, Fuerte and NASJAX, had the L993F mutation. Two additional amino acid changes, E434K and C764R, were found in all five of these strains. Further, each of five resistant individuals possessed only K434 and R764. An additional amino acid change, G330A, was detected in strains Malo and Fuerte, but not in Pinellis 214, Pinellis 417 or NASJAX. To determine if these amino acid changes were associated with knockdown resistance to pyrethroids, we determined sequences in the corresponding regions of the first five knocked-down individuals of all five strains and a susceptible strain, Orlando. The sequences from all susceptible individuals contained E434, C764 and G330, identical to those in the *para* sequence of the susceptible CSMA strain. The fact that the two amino acid changes (E434K and C764R) were detected only in the resistant individuals, but not in any susceptible individuals of any tested strain, strongly implicates 100% linkage of the two new mutations with the L993F mutation and their involvement in the knockdown resistance. However, susceptible individuals (the first five knocked-down) of the Malo and Fuerte strains also contained the G330 to A330 change, indicating that this change is not associated with *kdr* and more likely reflects neutral polymorphism between populations.

We then sequenced the remaining coding region of *para* from the five *kdr* individuals of the Malo strain, which exhibited the highest level of resistance. Compared with Para^{CSMA}, we found two additional amino acid changes, D58G and P1880L, in the Malo strain. However, we did not detect these two changes in the first five knocked-down individuals of Malo, the susceptible strain, Orlando, the first five and last five knocked-down individuals of Pinellis 417 (a high level of knockdown resistance) and Aves (a modest level of knockdown resistance) strains. All had D and P at the corresponding positions, like Para^{CSMA}. It is possible that the D58G and P1880L mutations detected in Malo are associated with the very high level of *kdr* in this strain. This needs to be tested in the future.

The D58G and P1880L mutations are located at the nitrogen and carbon termini, respectively. The E434K and C764R mutations are located in the first linker connecting domains I and II. E434K is situated close to the beginning of the linker region, whereas C764R is near the end of the linker (Fig. 1). The amino acid substitutions are quite drastic, especially from a negatively charged glutamic acid (E) to a positively charged lysine (K), from a cysteine (C) with a sulfhydryl group (highly reactive) to a positively charged arginine (R). Comparison of the deduced amino acid sequences of various sodium channel proteins in the regions where the four novel mutations reside reveals several intriguing features (Fig. 2). First, K434 detected in *kdr* cockroaches was found in all known sodium channel proteins of vertebrates. In contrast, E434 is conserved among insect Para and the squid sodium channel proteins (Fig. 2). It is known that mammals are more tolerant of pyrethroid insecticides compared with insects, often by several orders of magnitude (Elliott, 1976). More recently, Warmke et al. (1997) showed that rat brain type IIA sodium channel α -subunit was 100 times less sensitive to pyrethroids than the wild-type Para sodium channel. Our results, in the context of these findings, reveal a potential site in sodium channel proteins that may be involved in the selective toxicity of pyrethroid insecticides between mammals and insects. Secondly, C764 is conserved among all known sodium channel proteins, suggesting a potential role of C764 in sodium channel function. Furthermore, the D58G mutation is located at the nitrogen terminus. All insect Para proteins have an aspartic acid (D) at the corresponding position, while the rest of the channels in Fig. 2 (including ones not shown, see Goldin, 1995) all possess a glutamic acid (E), a conserved amino acid substitution, except for SNS which has a glycine (G) at this position. SNS belongs to a group of sodium channel proteins (Nav1.8) that are tetrodotoxin-resistant expressed primarily in small-diameter sensory neurons of the dorsal root ganglion and trigeminal ganglion (Goldin,

1999). It would be interesting to test the sensitivity of SNS channels to pyrethroid insecticides.

So far, the four additional *kdr*-associated mutations appear to coexist with the L993F mutation and have been found only in highly resistant strains. We sequenced *para* cDNA from a strain (Swine) that lacks the L993F mutation and exhibits only a modest level of *kdr* (Table 2). However, none of the additional mutations were detected in the Swine strain. These mutations also were not found in a *kdr* strain (Aves) that contained the L993F mutation (Valles et al., 2000) and exhibited only a moderate level of resistance (Table 2). It is important to note that Ectiban-R was selected from a DDT-resistant strain obtained in the late 1960s and the L993 to F993 mutation could be the result of DDT selection. We suspect that the four novel *kdr*-associated *para* gene mutations in the recently field-collected cockroach strains may have resulted from intensive pyrethroid applications since the 1970s. These mutations, together with the pre-existing L993F mutation, may be responsible for the high level of *kdr* resistance to pyrethroids in the German cockroach. They could be the counterparts of the second mutation (M to T in the linker connecting IIS4 and IIS5) in the super-*kdr* house fly. We are currently analyzing the mutant Para channels expressed in *Xenopus* oocytes to examine channel properties and sensitivity to pyrethroid insecticides. These novel mutations may directly or indirectly alter pyrethroid binding affinity. Alternatively, they may modify channel gating kinetics in a manner that counteracts the action of pyrethroids. Pyrethroids slow the kinetics of both activation and inactivation gates, resulting in prolonged opening of individual sodium channels (Narahashi, 1988). Pyrethroids also cause a shift of the channel activation and inactivation in the hyperpolarizing direction. Thus, a shift of the voltage dependence of activation or inactivation to more depolarizing membrane potentials could counteract (antagonize) the action of pyrethroids. It has been shown that voltage dependences of activation and inactivation were shifted significantly in the depolarization direction in the primary neurons isolated from a pyrethroid-resistant *Heliothis virescens* strain carrying the valine to methionine mutation in IS6 (Lee et al., 1999). Characterization of these novel mutations in an in vivo expression system, such as the *Xenopus* oocyte system, will significantly improve our understanding of the molecular basis of the *kdr* mechanism and the molecular interaction between pyrethroid insecticides and sodium channels.

Acknowledgments

The authors thank Drs Noah Koller and Jianguo Tan for critical review of the manuscript. The work was supported by grants from NSF (IBN 9696092 and IBN 98-08156) and NIH (08-R1GM57440A).

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Fig. 1.

kdr-associated mutations in the German cockroach Para sodium channel. The schematic diagram of the Para sodium channel indicates four homologous domains (I–IV), each with six transmembrane segments (S1–6). L993F was previously identified in a *kdr* strain (Ectiban-R) (Dong, 1997). Four additional mutations (D58G, E433K, C764R and P1880L, identified in this study) are associated with high levels of resistance.

Fig. 2.

Alignment of amino acid sequences of regions where *kdr*-associated *para* mutations reside among sodium channel α -subunits from various species and tissues. Sequence alignment was carried out with the entire amino acid sequences of Para^{CSMA} and other sodium channel α -subunits using the Clustal method (DNASTAR). D⁵⁸, E⁴³³, C⁷⁶⁴ and P¹⁸⁸⁰ in Para^{CSMA} (wild-type Para) and the corresponding amino acid residues in other sodium channel sequences are boxed. Indicated above each box is the amino acid substitution found in the *kdr* cockroach Para. The first three sequences are insect Para protein sequences (accession numbers): cockroach Para^{CSMA} (U73583; Dong, 1997), *Drosophila* Para (M24285; Loughney et al., 1989) and house fly Vssc1 (U38813; Ingles et al., 1996). GFLN1 is from the giant axon of the squid *Loligo opalescens* (L19979; Rosenthal and Gilly, 1993); Eel is from *Electrophorus electricus* (M22252; Noda and Numa, 1987); Nas is from rabbit Schwann cell (U35238; Belcher et al., 1995); PN1 is from rat peripheral neurons (U79568; Toledo-Aral et al., 1997); NaNG is from dog nodose ganglion neurons (U60590; Chen et al., 1997); Rat IIA is from rat brain (X 61149; Auld et al., 1988); rH1 is from rat cardiac muscle (M27902; Rogart et al., 1989); μ 1 is from rat skeletal muscle (M26643; Trimmer et al., 1989); HBA is from human brain (M94055; Ahmed et al., 1992); SNS is from rat sensory neurons (X92184; Akopian et al., 1996).

Table 1

Oligodeoxynucleotide primers used in PCR

Primer no. ^a	Nucleotide sequence (5' to 3')	Amino acid position	Region in Para
<i>Primers used to amplify cDNAs encoding mainly transmembrane regions</i>			
1	gcgaaccacagcagcaatg	5'-UTR	N-terminus-IS5
2	gcatgccagttctcatcattca	303-309	
3	gctccgagcttgaagactgtc	232-238	IS5-IS6
4	gctagttctgctgctgcta	464-470	
5	gatgacgagggtccaacagtta	739-745	IIS1-IIS6
6	cttggtggttcattgtc	1025-1030	
7	tgaggacgtcatgatgcagaatatcc	1228-1235	IIIS1-IIIS6
8	tctagcgaccctcctgcc	1546-1552	
9	gcagccaatcagggaacgaacatc	1504-1511	IIIS6-IVS6
10	caaagcatccaaatgtccacac	1915-1922	
<i>Primers used to amplify cDNAs covering the entire coding region</i>			
11	gcgaaccacagcagcaatg	5'-UTR	
12	gctccatcatcactgtctgctgac	686-694	
13	gctccatcatcactgtctgctgac	623-629	
14	gctccttggactcttctgtctc	1094-1101	
15	gctgacaatgaaaccaacaagatt	1025-1032	
16	tctagcgaccctcctgcc	1546-1552	
17	gcagccaatcagggaacgaacatc	1504-1511	
18	aatcaagcgaagatgtgag	3'-UTR	

^aThe odd number primers are sense primers, whereas the even number primers are antisense primers.

Table 2

Knockdown times and *para* mutations among different German cockroach strains

Strain	<i>para</i> mutation				Time to knockdown (min)		
	L993F ^a	D58G	G330A	E434K	C764R	P1880L	Last five ^b
CSMA	L	D	G	E	C	P	<20 ^c
Orlando	L	D	G	E	C	P	<18 ^c
Ectiban-R	F	D	G	E	C	P	45–70 ^c
	S/R ^d	S/R	S/R	S/R	S/R	S/R	
Swine	L/L ^e	f	–	–/E	–/C	–	15–20
Aves	–/F	D/D	–	–/E	–/C	P/P	17–23
Malo	L/F	D/G	A/A	E/K	C/R	P/L	50–81
Pinellis 214	L/F	–	–	E/K	C/R	–	8–37
Pinellis 417	L/F	D/D	–	E/K	C/R	P/P	11–29
Fuerte	L/F	–	A/A	E/K	C/R	–	14–48
NASJAX	–/F	–	–	E/K	C/R	–	15–30

^aData taken from Dong et al. (1998) and Valles et al. (2000).

^bTime at which the first five or the 45th individual of 50 cockroaches was knocked down in the cypermethrin residue bioassay.

^cTime at which all 50 individuals were knocked down.

^dPhenotype: susceptible (S; the first five knocked-down individuals)/resistant (R; the last five knocked-down individuals).

^eGenotype: the amino acid residue in the first five knocked down individuals/the amino acid in the last five knocked down individuals.

^fNot examined.