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Toxicological, Electrophysiological, and Molecular Characterisation of Knockdown Resistance to Pyrethroid Insecticides in the Diamondback Moth, *Plutella xylostella* (L.)

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Nerve insensitivity was shown to be a major cause of high pyrethroid resistance in a Taiwanese strain of the diamondback moth, *Plutella xylostella.* Initial evidence for this type of target site insensitivity, also termed knockdown resistance or *kdr,* was provided by nonsynergizable cross-resistance to a range of pyrethroids and DDT and an incompletely recessive autosomal inheritance of the resistance trait. This was corroborated by using a larval neuromuscular preparation to assess spontaneous miniature excitatory postsynaptic potentials (mEPSPs) and evoked EPSPs in response to varying concentrations of the pyrethroid deltamethrin. Intracellular recordings revealed a pyrethroid-induced increase in mEPSP activity and a decline in the EPSP amplitude; responses were induced only at considerably higher concentrations in resistant larvae when compared to larvae of a susceptible standard strain. These findings were supported by the detection of two amino acid changes in part of the *para*-type voltage-sensitive sodium channel (the primary target site of pyrethroids) of the resistant strain. One of these mutations, a leucine to phenylalanine replacement in transmembrane segment 6 of domain II, has previously been shown to correlate with *kdr* in the house fly, *Musca domestica,* and German cockroach, *Blattella germanica.* ©1998 Academic Press

INTRODUCTION

Plutella xylostella (L.) (Lepidoptera: Yponomeutidae), a major cosmopolitan pest of brassicas, represents one of the extreme cases of insecticide resistance in arthropods (1, 2). Detoxification by microsomal P450-dependent monooxygenases has been considered the principal mechanism of pyrethroid resistance in *P. xylostella* but other mechanisms are also thought to be important (2, 3). Considerable indirect evidence has been accumulated for a widespread occurrence of nerve insensitivity in *P. xylostella* based on (a) cross-resistance between pyrethroids and DDT (4, 5), (b) lack of synergism by inhibitors of possible detoxifying enzymes such as PB (6, 7), and (c) partially recessive inheritance of pyrethroid resistance (8, 9) similar to the inheritance of nerve insensitivity in house flies (10). The first direct evidence came from electrophysiological tests with pyrethroid-resistant Japanese strains of *P. xylostella*, which demonstrated a delayed response of the central nerve cord to fenvalerate (11). However, the importance of nerve insensitivity in pyrethroid resistance in this insect has remained in doubt (2).

Toxicological and electrophysiological studies have demonstrated the presence of nerve insensitivity in other pyrethroid-resistant insects including species of Lepidoptera (12–15), Diptera (16–20), Coleoptera (21), and Dictyoptera (22). The voltage-sensitive sodium channel of nerve membranes is considered to be the primary target of pyrethroid insecticides and DDT, and reduced nerve sensitivity is considered to be one of the major mechanisms responsible for resistance to these compounds in insects (23). This type of resistance was first observed in house flies (*Musca domestica*) and termed

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knock-down resistance (*kdr*)[2] (24, 25). Since then different *kdr* alleles have been described in the house fly and mapped to autosome 3, including both *kdr* itself and the more potent *super-kdr* allele (10, 26, 27). It is thought that nerve insensitivity in flies possessing *kdr* and *super-kdr* is based on changes to the sodium channel leading to reduced binding of these insecticides (28). This is supported by recent molecular cloning studies of the *para*-type sodium channel gene in *M. domestica* (29) and the German cockroach *Blattella germanica* L. (30) that have identified two amino acid substitutions in the channel sequence that correlate with the *kdr* resistance phenotypes.

The present study used a highly pyrethroid-resistant *P. xylostella* strain from Taiwan. Previous studies using synergists and model substrates had indicated that enhanced microsomal P450-dependent monooxygenases were an important mechanism of pyrethroid resistance in this strain (31, 32). We now report evidence for the presence of strong nerve insensitivity, based on toxicological, electrophysiological, and molecular studies.

MATERIALS AND METHODS

Insects

Two *P. xylostella* strains were maintained in the laboratory on Chinese cabbage (*Brassica chinensis* var. *pekinensis* (Rubr.) Sun.) at 22°C without selection pressure. The susceptible Rothamsted strain has been in laboratory culture for more than 30 years. The pyrethroid-resistant FEN strain (formerly FP, C.-N. Sun, personal communication) was obtained in 1995 from C.-N. Sun, National Chung-Hsing University, Taichung, Taiwan. FEN originated from a heterogeneous Taiwanese field population, collected in 1983, which was subsequently selected with fenvalerate in the laboratory (6). After arrival in the UK, every generation was tested with fenvalerate and bioresmethrin to monitor for any changes in resistance levels.

Bioassays

Insecticide activity against fourth instar *P. xylostella* larvae was assessed by topical application of 0.5μl droplets of technical compound in acetone. The maximum dose of insecticide applied was 10 μg/larva with the exception of fenvalerate, for which a dose of 100 μg/larva was used with the resistant strain. Treated larvae were supplied with Chinese cabbage leaf discs and mortality was assessed after 5 days at 22°C. Tests were conducted at least twice except for those with synergists and on F_1 progeny. Three replicates with 10 larvae each were used for each dose in LD_{50} studies. However, bioassays with the resistant FEN strain were mostly restricted to single-dose tests with 10 μg/larva. Synergists (0.5 μl/larva) were applied topically to FEN 30–60 min before the insecticide. Two to three replicates of 10 larvae were used for each single-dose test.

Insecticides and Synergists for Bioassays

Insecticides and synergists used for bioassays were synthesised at Rothamsted unless otherwise indicated, and were of technical grade or purer. The following synergists were used: PB and PBX (both supplied by Endura Spa, Bologna, Italy), TCPB (supplied by N. W. Forrester, New South Wales Agriculture, Australia), Niagara 16824, and FDMC.

Interstrain Cross

Reciprocal crosses between Rothamsted and FEN insects were achieved by mass mating virgin adults of each sex, obtained by sexing fourth instar larvae. Single LD_{50} tests were performed

[2] Abbreviations used: EPSP, excitatory postsynaptic potential; FDMC, 2,2-bis(4-chlorophenyl)-1,1,1-trifluoroethanol; FEN, pyrethroid-resistant *Plutella xylostella* strain; *kdr,* knockdown resistance; LEP1, LEP2, internal *P. xylostella*-specific sodium channel oligonucleotide primers; MAP, muscle action potential; mEPSP, miniature excitatory post-synaptic potential; PB, piperonyl butoxide; PBX, a mixture of 5-[2-(2-butoxyethoxy)ethoxymethyl]-1,3-benzodioxole,5,6-di[2-(2-butoxyethoxy)ethoxymethyl]-1,3-benzodioxole and 4,5,6-tri-[2-(2-butoxyethoxy)ethoxymethyl]-1,3-benzodioxole; PCR, polymerase chain reaction; RF, resistance factor; TCPB, 1,2,4-trichloro-3-(2-propargyl-)benzene; VIL, ventral internal lateral muscles.

against the resulting F_1 fourth instar progeny by topical application as described above.

Neurophysiological Assay

Fourth instar larvae, starved for 2–3 days at 4°C to reduce fat deposits which obscure underlying muscles, were pinned anteriorly and posteriorly in a 1-ml-well cut from Sylgard (Dow Corning). Following the introduction of saline ((mM) NaCl (5), KCl (39), $CaCl_2$ (6), $NaHCO_3$ (10), $MgCl_2$ (40), Hepes (10), Sucrose (93), pH 6.8), larvae were decapitated, cut open along the middorsal line, and the body walls were pinned flat. Viscera were removed to display the body wall musculature and the ventral internal longitudinal muscles (VIL) 6 and 7 (33). Excitatory postsynaptic potentials (EPSPs) were evoked by stimulation using a suction electrode attached to the segmental ganglion. Square current pulses, 0.5 ms duration at 1 Hz, were applied to the ganglion and the intracellular EPSP was measured in VIL muscles located in the segment posterior to the ganglion. Muscle cells were impaled with glass microelectrodes (15–20 mol wt) filled with 4 M potassium acetate. Signals were amplified with an Axoclamp 2A amplifier (Axon Instruments), digitised using a Sony PCM 701, and recorded on videotape for subsequent analysis. Spontaneous miniature EPSPs (mEPSPs) and evoked EPSPs were displayed and analysed using the computer package Axotape (Axon Instruments) mounted on a PC.

Following a 10-min control incubation period in saline containing 0.1% v/v acetone, increasing concentrations of deltamethrin in saline containing 0.1% acetone were continuously perfused over the preparation at a rate of approximately 2 ml/min. Concentrations from 10^{-12} to 10^{-6} M in 100-fold increments were perfused for 10 min each. Experiments were carried out at room temperature (21–24°C). New baths and dissection pins were used for each preparation and the perfusion system was flushed with 100 ml of 100% ethanol between each experiment to avoid accumulation of residues of deltamethrin.

Statistical Analysis

Bioassay mortality data were subjected to probit line analysis using the POLO-PC program. The statistics package, MLP (Maximum Likelihood Program), was used to analyse the electrophysiological results. MLP fitted normal distributions to both susceptible and FEN strains on a $\log_{10}$ scale. "Parallel" samples were then fitted and the standard deviation was constrained to be the same in each sample, which is equivalent to standard parallel probit line analysis.

Analysis of Sodium Channel Gene Sequences

Six fourth instar larvae of each strain were ground to a fine powder in liquid nitrogen and homogenised in 0.5 ml 4 M guanidinium thiocyanate. Total RNA was recovered by extracting the homogenate three times with equal volumes of phenol/chloroform, and the RNA was precipitated with cold ethanol. The RNA (1–2 μg) was reverse transcribed into single-strand cDNA using the enzyme Superscript II (Life Technologies Ltd.) and oligo(dt) primer (200 ng) under the supplier's recommended conditions. The cDNA was used as template for two rounds of PCR with degenerate sodium channel primers as described elsewhere (34). The 350-bp sodium channel fragments amplified from each strain were cloned into a T-tailed plasmid vector (Invitrogen) and sequenced in both strands using the vector M13 forward and reverse primers on an Applied Biosystems 373 automated sequencer. To confirm that these cloned sequences did not contain *Taq* polymerase "errors," two internal *P. xylostella*-specific primers (LEP1 and LEP2, see Fig. 3) were made and used to resequence directly the ethanol-precipitated PCR fragments. This direct sequencing of PCR fragments overcomes the problem of *Taq* mistakes that is often encountered in the sequencing of individual cloned fragments. For the analysis of individuals from the neurophysiological assay, the larvae were frozen in liquid nitrogen at the end of the assay and stored at −80°C. RNA was extracted using a scale-down of the method above and, following PCR, the fragments were direct-sequenced using primers

LEP1 and LEP2. Sequences were processed and analysed using Staden (35) and Wisconsin GCG (36) software packages.

RESULTS

Bioassays

The FEN strain was strongly resistant to a range of pyrethroid insecticides (Table 1). Resistance factors (RF) against the benzylfurylmethyl pyrethroids bioresmethrin and cismethrin were 1700 and 5000, respectively, while α-cyano-phenoxybenzyl pyrethroids were so strongly resisted that no LD_{50}s could be estimated (RF >10,000). Even with bioresmethrin and cismethrin, LD_{50}s could only be estimated by being in the region of the highest dose applied (10 μg/insect). Mortality caused by 100 μg fenvalerate never exceeded 8% and averaged 3%. DDT was completely ineffective against larvae of the FEN strain (Table 1).

In contrast to previously published work there was still a very high level of resistance in the presence of the microsomal monooxygenase inhibitor PB (Table 2). 100 μg/larva fenvalerate applied after PB gave only 59% mortality of FEN larvae, compared to a LD_{50} of 0.003 μg/larva for the susceptible strain. PBX, Niagara 16824, *m*-nitro propargyl ether, and TCPB were even less effective as synergists. PB also had only a marginal synergistic effect on the susceptibility of larvae to bioresmethrin. In agreement with earlier work (5), no synergism of DDT was achieved by PB or the DDT dehydrochlorinase inhibitor FDMC. The inhibitors applied alone resulted in negligible mortality of FEN larvae (Table 2).

Crossing FEN with the susceptible Rothamsted strain reduced the RF to fenvalerate from >33,000 to 20 in the F_1 insects, and almost eliminated resistance to bioresmethrin (Table 3). Larvae derived from reciprocal crosses did not differ significantly in their susceptibility to bioresmethrin. It can therefore be concluded that pyrethroid resistance in FEN is an autosomally inherited, largely recessive trait. No decrease in the degree of resistance of FEN to fenvalerate and bioresmethrin was observed over 24 generations in the laboratory at Rothamsted, implying a high level of homozygosity for the resistance gene(s) in this strain.

Neurophysiology

Resting membrane potentials of VIL 6 and 7 in Rothamsted (-62.03 ± 5.6 mV, $n = 40$) and FEN (-61.43 ± 6.8 mV, $n = 43$) larvae did not differ significantly ($P > 0.95$, one-way ANOVA) and were stable during the period of

TABLE 1

Susceptibility to Pyrethroids and DDT of Larvae of the Susceptible Rothamsted Strain and the Resistant FEN Strain of Plutella xylostella

		Strain			
		Rothamsted		FEN	
Compound	Alcohol moiety	LD_{50}, μg/larva (95% FL)	Slope ± SE	LD_{50}, μg/larva	RF[a]
Bioresmethrin	5-benzyl-3-furylmethyl	0.006 (0.0051–0.0067)	2.21 ± 0.116	ca. 10	1700
Cismethrin	5-benzyl-3-furylmethyl	0.002 (0.0016–0.0040)	2.12 ± 0.329	ca. 10	5000
Fenvalerate	α-cyano-3-phenoxybenzyl	0.003 (0.0026–0.0033)	4.15 ± 0.471	100 μg = 3% kill	>33000
Deltamethrin	α-cyano-3-phenoxybenzyl	0.001 (0.0007–0.0011)	1.97 ± 0.210	10 μg = 6% kill	>10000
DDT	—	0.92 (0.64–1.59)	1.75 ± 0.291	10 μg = 1% kill	>10

[a]Resistance factor, LD_{50} of FEN/LD_{50} of Rothamsted strain.

TABLE 2
Effect of a Range of Enzyme Inhibitors on the Susceptibility of Larvae of the FEN Strain of Plutella xylostella to Pyrethroids and DDT

Insecticide	Dose (μg/larva)	Inhibitor[a]	Dose (μg/larva)	Mortality (%)		
				Insecticide only	Inhibitor only	Inhibitor plus insecticide
		Microsomal monooxygenase inhibitors				
Fenvalerate	100	PB	5	0	0	58.6
		PBX	5	0	5.0	20.0
		Niagara 16824	5	0	0	25.0
		m-nitro propargyl ether	5	0	3.3	10.0
		TCPB	5	0	0	3.3
Bioresmethrin	10	PB	1	55.2	0	77.8
DDT	10	PB	1	0	0	0
		DDT-dehydrochlorinase inhibitor				
DDT	10	FDMC	1	0	0	0

[a]Inhibitors were applied 30–60 min prior to the insecticides.

the experiment, with the exception of a slow depolarisation of 5–10 mV which accompanied the effects of deltamethrin in some preparations.

In untreated muscles from both Rothamsted and FEN strain insects, the membrane potential showed only slight fluctuations (<0.1 mV) peak to peak and mEPSPs were not obvious, although occasional depolarisations up to 0.2 mV were recorded. Perfusion of deltamethrin over Rothamsted larvae resulted in a massive sustained increase in the frequency of spontaneous mEPSP within 10 min. The response was seen as large (1–2 mV) fluctuations in the membrane potential (Fig. 1a inset). The high frequency mEPSP discharge continued for 5–10 min after which time the membrane potential returned to a steady baseline (Fig. 1a). In contrast, FEN strain larval muscle was much less affected by application of deltamethrin, with the lowest concentration of deltamethrin needed to produce a response in FEN larvae being 4 orders of magnitude higher than that which affected Rothamsted larvae. The maximum concentration used (10^{-6}M) produced a response in only 40.9% of FEN larvae tested (Table 4; Fig. 1b).

Stimulation of the segmental nerve resulted in a depolarising response recorded in VIL 6 or 7, comprising an EPSP and a muscle action

TABLE 3
Susceptibility to Fenvalerate and Bioresmethrin of Larvae of F_1 Crosses between the Susceptible Rothamsted Strain and the Resistant FEN Strain of Plutella xylostella

	LD_{50}, μg/larva (95% FL)	Slope ± SE	RF[a]
Fenvalerate			
FEN males × Rothamsted females	0.064 (0.046–0.085)	1.96 ± 0.263	20
Bioresmethrin			
FEN males × Rothamsted females	0.019 (0.010–0.028)	1.98 ± 0.438	3
Rothamsted males × FEN females	0.021 (0.013–0.034)	1.23 ± 0.291	4

[a]Resistance factor, LD_{50} of F_1 progeny/LD_{50} of Rothamsted strain (Table 1).

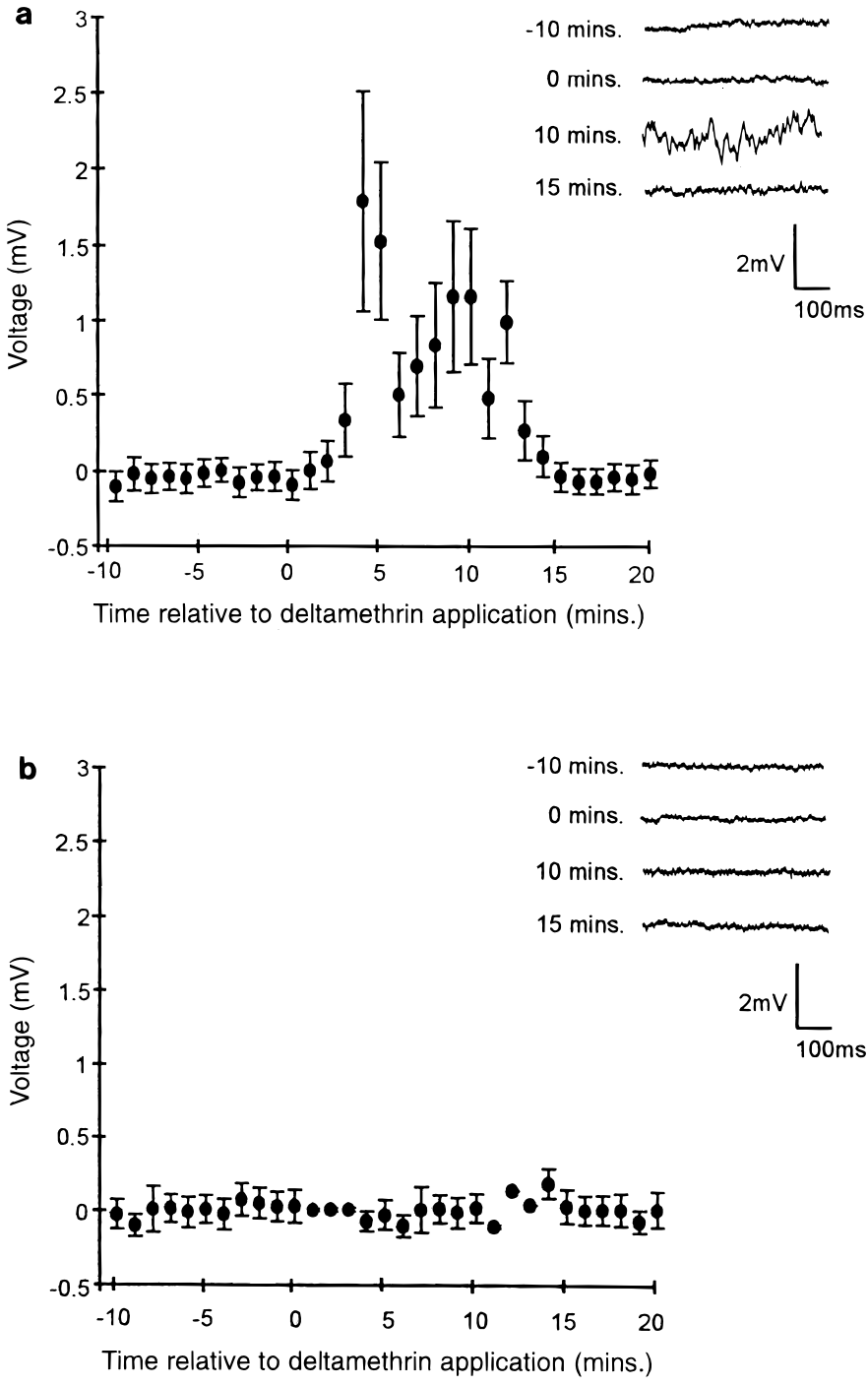

FIG. 1. *Spontaneous miniature excitatory postsynaptic potentials (mEPSPS) recorded intracellularly from ventral internal longitudinal (VIL) muscles of a fourth instar Plutella xylostella larva stimulated via the segmental ganglion at 1Hz. 1a shows the time course of the increase in mEPSP activity following the application of 10^{-12} M deltamethrin to a Rothamsted larva. Inset are recordings of the membrane potential of a VIL muscle fibre at various times relative to the onset of deltamethrin perfusion showing the increase in spontaneous mEPSP activity after 10 min. The plotted data show the mean peak amplitude of mEPSPs sampled over 500 ms. Each point represents the mean ±SD of five periods of mEPSP activity at 1-s intervals. Resting membrane potential = −57 mV. 1b shows the action of 10^{-6} M deltamethrin on mEPSPs from a FEN strain larva expressed as for 1a. Resting membrane potential = −58mV.*

TABLE 4

Distribution (%) of Neuronal Responses[a] to a Range of Deltamethrin Concentrations in the Cumulative Electrophysiological Assay Using Fourth Instar Larvae of the Susceptible Rothamsted Strain (n=40) and the Pyrethroid-Resistant FEN Strain (n=43) of Plutella xylostella[b]

Deltamethrin (M)[c]	Strain[d]	
	Rothamsted	FEN
10^{-12}	32	0
10^{-10}	82	0
10^{-8}	100	14
10^{-6}		41
No response at 10^{-6}	0	59
EC_{50}[e]	3.98×10^{-12} M	1.66×10^{-6} M

[a]Neuronal response is defined as the simultaneous increase in mEPSPs and decline in evoked EPSP seen in Figs. 1a and 2a.

[b]Larvae challenged with acetone at 1 μl/ml did not display any effects.

[c]Larvae were not challenged at concentrations above 10^{-6} M deltamethrin.

[d]Twenty-two larvae were tested for each strain.

[e]Difference between EC_{50}s highly significant, $\chi^2 = 52.03$, 1 df.

potential (MAP), (Figs. 2a and 2b). The mean amplitude of this response was 56.51 ± 7.81 mV ($n = 40$) for Rothamsted insects and 54.8 ± 10.19 mV ($n = 43$) for FEN larvae. Reduction of the stimulus resulted in failure of the EPSP to reach threshold for MAP generation. Addition of deltamethrin to Rothamsted larvae was followed by a reduction of the amplitude of the EPSP which fell below threshold for the MAP and then gradually declined to zero (Fig. 2a). FEN strain larvae were again much less responsive to deltamethrin (Table 4; Fig. 2b). In all cases the action of deltamethrin was to produce both a large increase in mEPSP frequency and a complete irreversible block of the EPSP and MAP.

To compare the sensitivity of Rothamsted (40 larvae) and FEN (43 larvae) to deltamethrin, neuromuscular preparations from each strain were perfused with increasing concentrations (100-fold increments) of the pyrethroid. The insecticide was continuously perfused for 10 min at each concentration, and the concentration required to induce both an increased frequency of mEPSPs and failure of the evoked EPSP was recorded. The data are shown in Table 4 and EC_{50} values were computed as described. The EC_{50} values were compared using the χ^2 test and found to be significantly different with the resistant FEN strain being 390,000-fold less susceptible to deltamethrin than Rothamsted ($P < 0.001$).

Analysis of Sodium Channel Gene Sequences

To investigate whether the FEN strain contained either of the sodium channel mutations associated with *kdr* resistance phenotypes in the house fly (29) and German cockroach (30), the domain II region of the *para*-type sodium channel to which these mutations were localised was PCR amplified and sequenced from both the Rothamsted and FEN strains. Degenerate PCR primers designed against highly conserved sequences of the sodium channel, which enable the selective amplification of the IIS4-IIS6 region of the *para* sodium channel gene from a range of insect species (34), were used to amplify fragments of the expected size (350 bp) from each strain, which were cloned and sequenced. cDNA was used in preference to genomic DNA as the template for PCR because the *para* gene contains several introns in this region ((37) and our unpublished data), one of which disrupts the 3′ primer site. The cloned fragments encoded amino acid sequences with close identity to the corresponding region of the house fly (94%) and cockroach (89%) *para*-type sodium channels. The nucleotide and amino acid sequences of the Rothamsted and FEN strains are shown in Fig. 3 (EMBL/Genbank Accession Nos. AJ223278 and AJ223279). The sequences contain two base changes, both of which cause amino acid substitutions in the encoded channel sequences (highlighted in Fig. 3). These changes were confirmed by direct sequencing of the PCR fragments using internal *P. xylostella*-specific primers (LEP1 and LEP2, Fig. 3) and so are not the result of *Taq* polymerase errors. Moreover, one of the changes is exactly the same leucine (CTT, Rothamsted) to phenylalanine (TTT, FEN) substitution in the IIS6 transmembrane

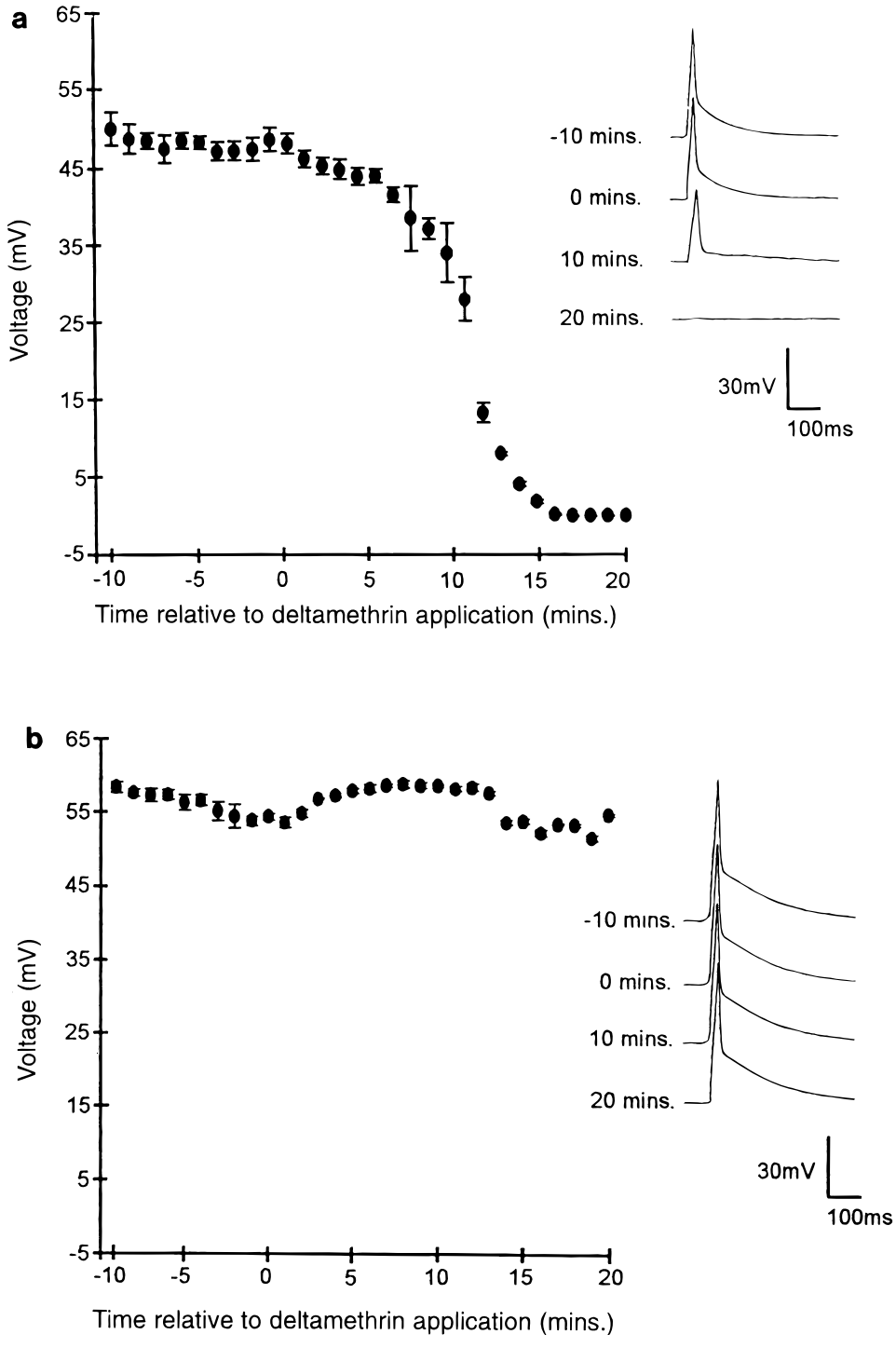

FIG. 2. *Neurally evoked excitatory postsynaptic potentials (EPSPs) recorded intracellularly from VIL of the same larvae as shown in Fig. 1. 2a demonstrates the time course of block of the neurally evoked EPSP following application of 10^{-12} M deltamethrin to a Rothamsted larva. Inset shows the decline in amplitude of the EPSP 10 min after addition of deltamethrin followed by complete abolition of the response after 20 min. Each point on the graph represents the mean peak amplitude of five consecutive EPSPs ± SD sampled at 1-min intervals. Note the variable amplitude of the postsynaptic potential after 5–10 min due to failure of the EPSP to evoke a muscle potential in some responses following addition of deltamethrin. 2b shows the action of 10^{-6}M deltamethrin on mEPSPs from a FEN strain larva expressed as for 2a.*

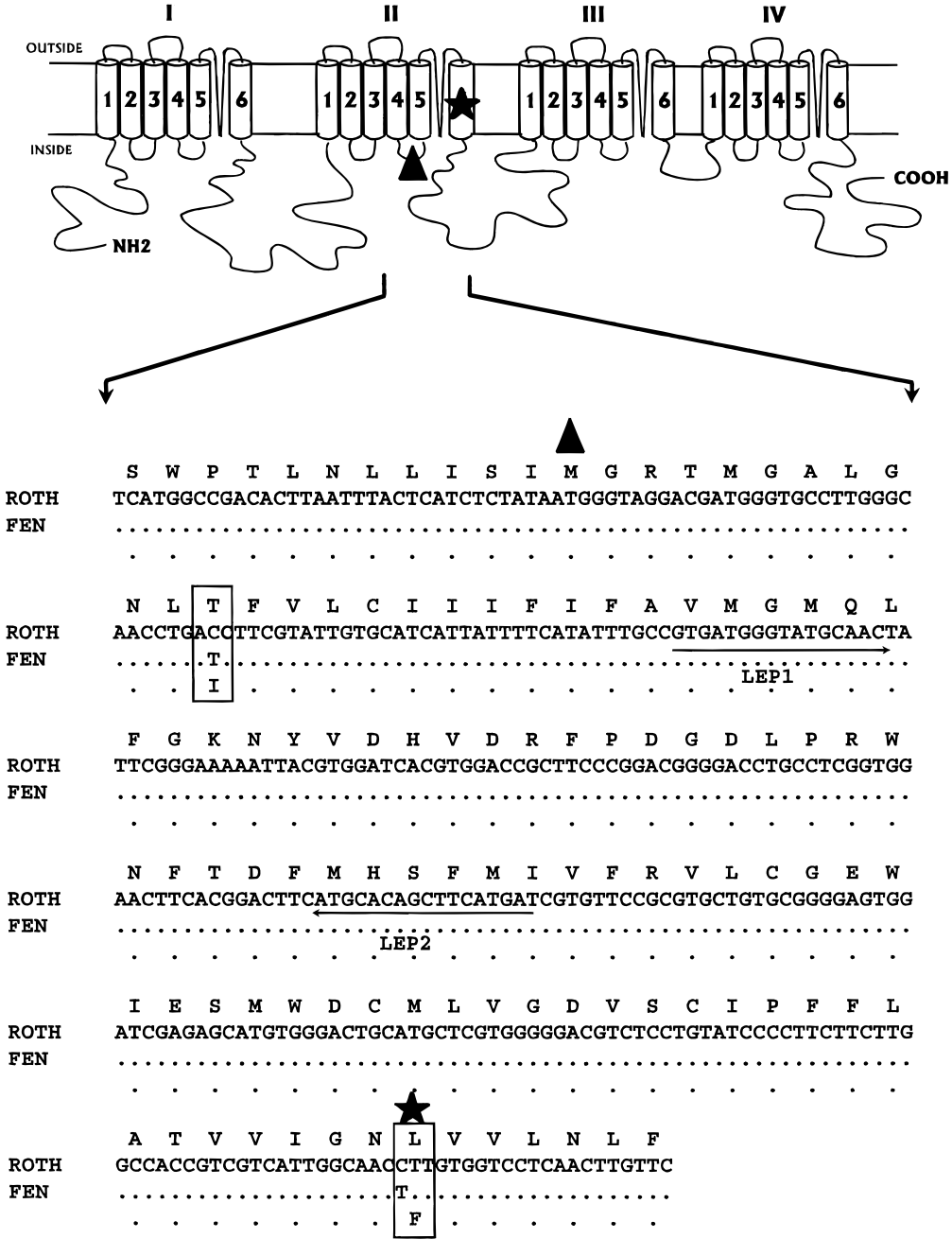

FIG. 3. *(Top) Diagram of the sodium channel showing the four main domains (I–IV) and proposed membrane folding of the transmembrane segments (S1–S6) within each domain. (Bottom) Nucleotide and deduced amino acid sequences of the IIS4–IIS6 region amplified from Rothamsted and FEN strains; only differences in the FEN sequence are shown. The two mutations in FEN are boxed. The LEP1 and LEP2 sequencing primers are shown as arrows, and the positions of the house fly kdr (leucine to phenylalanine, IIS6) and super-kdr (methionine to threonine, IIS4-S5) mutations are highlighted in the diagram and above the Plutella xylostella sequence.*

segment as previously reported for house fly and cockroach *kdr* strains. The other change, however, is different to that in *super-kdr* house fly strains, causing a threonine (ACC, Rothamsted) to isoleucine (ATC, FEN) substitution at the beginnning of the IIS5 segment (Fig. 3). Both mutations affect residues that are otherwise highly conserved in the known sodium channel sequences of vertebrates and invertebrates (Fig. 4). The association of these changes with the nerve-insensitive phenotype of the FEN strain was further confirmed by carrying out the PCR/sequence analysis on individual FEN larvae from the neurophysiological assay. Twelve larvae were sequenced, all of which were homozygous for the two changes described above.

DISCUSSION

The toxicological data presented demonstrate high levels of pyrethroid resistance in the FEN strain, with resistance factors for the type I pyrethroids bioresmethrin and cismethrin of 1700- and 5000-fold, respectively. Even higher resistance ratios (>10,000) were obtained with the type II pyrethroids fenvalerate and deltamethrin, which are characterised by an α-cyano side group. Previous work with the FEN strain has also reported extremely high levels of resistance to fenvalerate (6, 31). The effect of pyrethroids on the FEN strain was only slightly synergized by a range of metabolic inhibitors, which contrasts with the previous finding that pretreatment with PB reduced the LD_{50} for fenvalerate by over 18-fold (6). The latter finding was supported by 2- to 18-fold higher activity of monooxygenases in the FEN strain (7, 31, 32). The lack of synergism in our bioassays suggests that the metabolic resistance associated with this strain has been lost without any measurable decrease in resistance levels. Yu and Nguyen (38) also reported metabolic resistance to pyrethroids in *P. xylostella* to be less stable than target site insensitivity.

Several lines of evidence now point to nerve insensitivity as the major resistance mechanism in the FEN strain. α-Cyano compounds were more strongly resisted by the FEN strain than type I pyrethroids, a structure–activity relationship which follows the pattern previously described for *super-kdr* house fly strains (26, 39).

Nonsynergizable cross-resistance to DDT and pyrethroids as reported here is a further indicator for the presence of target site insensitivity since the sodium channel in nerve membranes is considered the primary target site for both DDT and pyrethoids. Nonsynergizable DDT resistance has also previously been reported for another Taiwanese strain of *P. xylostella* (5).

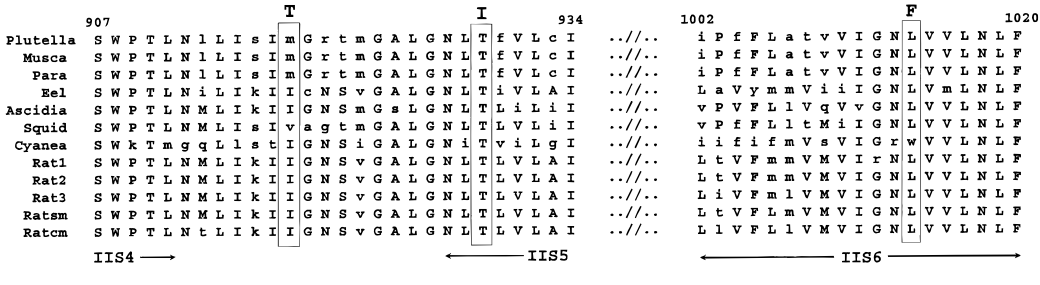

FIG. 4. *Alignment of sodium channel sequences from a range of vertebrates and invertebrates in the regions of the two FEN mutations showing the otherwise high conservation of these residues across species. The FEN changes (threonine to isoleucine; leucine to phenylalanine) are shown above the sequences, as is the house fly super-kdr mutation (methionine to threonine) which occurs in addition to the leucine to phenylalanine kdr mutation. Numbering is according to the house fly sequence of Williamson et al. (29). Sequences are from Plutella xylostella (Rothamsted strain), Musca domestica (susceptible), Drosophila melanogaster (para), Electrophorus electricus (eel), Halocynthia roretzi (Ascidia), Loligo bleekeri (squid), Cyanea capillata (Cyanea) rat brain type I, II, and III genes (Rat1,2,3), and the rat skeletal and cardiac muscle genes (Ratsm, Ratcm).*

The apparent lack of metabolic resistance from synergist studies is also supported by the crossing experiment which showed pyrethroid resistance in FEN to be a largely recessive trait showing no sex linkage. This is in agreement with all previous studies on the inheritance of strong pyrethroid resistance in *P. xylostella* (8, 9, 11, 40, 41) and is in line with the recessive nature of nerve insensitivity in the house fly (42). In contrast, the inheritance of monooxygenase-based pyrethroid resistance in another lepidopteran species, *Helicoverpa armigera*, was reported to be incompletely dominant (43).

The electrophysiological assay provided conclusive evidence for a very high level of nerve insensitivity (over 400,000-fold) to deltamethrin in the FEN strain. A similar assay was previously used by Irving *et al.* (44) who concluded that the increase in mEPSP frequency was sufficiently large to make intracellular recording from muscle fibres a practical method for routine neurophysiological assays with *P. xylostella.* Although preparations contained both neural and muscular elements, the pyrethroid-induced responses were thought to be a product of neural toxicity alone. While experiments on isolated axons have demonstrated differences between susceptible and resistant strains of house fly (45), experiments by Salgado *et al.* (46) revealed no change in muscle electrical properties. Nevertheless, results with neuromuscular preparations, especially changes in the mEPSP frequency, have been shown to correlate well with whole organism bioassays (46–48). Thus, the electrophysiological effects induced by pyrethroids in the present study are likely to reflect a primary action of the insecticide.

Identification of the leucine to phenylalanine substitution within the IIS6 segment of the *para*-type sodium channel of FEN insects further consolidates the association of this substitution with *kdr*-type resistance in a range of insect species. In addition to house flies (29) and cockroaches (30, 49), it has been found to be associated with resistance to pyrethroids and DDT in the mosquito, *Anopheles gambiae* (50) and peach-potato aphid, *Myzus persicae* (34). Moreover, a change in this leucine (to histidine rather than phenylalanine) residue has recently been reported in two pyrethroid-resistant field populations of the tobacco budworm, *Heliothis virescens* (51). The consistent identification of single-base mutations at this position provides overwhelming evidence that the consequent changes at this particular leucine residue are indeed responsible for causing the *kdr*-type nerve insensitivity phenotype. The significance of the second mutation (threonine to isoleucine) within the FEN sequence is, however, less clear since this substitution has not been reported in the channel sequences of any of the above insect species. It could represent a polymorphism with no relevance to resistance. However, two lines of evidence suggest that this mutation may play an important role in enhancing the resistance phenotype of the FEN strain. Firstly, it is located just 11 amino acids downstream of the methionine to threonine substitution of *super-kdr* house flies, which is strongly implicated in enhancing levels of pyrethroid resistance in that species (29). While this enhanced resistance is observed across the entire class of pyrethroids and to DDT, in house flies it is most pronounced for α-cyano pyrethroids (39). The resistance profile of the FEN strain resembles that of *super-kdr* house-flies in that it not only displays a high level of resistance to all the compounds tested (>1000-fold), but there is also a clear enhancement against the two α-cyano compounds, fenvalerate and deltamethrin. Indeed, more than 90% of the FEN larvae routinely survived the highest dose of these compounds that could be applied in bioassays. Hence, it is tempting to speculate that the threonine to isoleucine change has a similar effect to the nearby methionine to threonine mutation of *super-kdr* house flies. Secondly, the high degree of conservation of this threonine residue in sodium channels from other species (Fig. 4) argues against this being a coincidental polymorphism between strains. Threonine is found at this position in all known sodium channel sequences, both vertebrate and invertebrate, suggesting that it is under strong selective pressure and is likely to be important for the structural or functional properties of this region of

the channel. This constraint would only be lifted if the mutation conferred an alternative advantage in the face of a new selection pressure, e.g., that for resistance to insecticides. We are currently investigating the distribution of this mutation in pyrethroid-resistant *P. xylostella* strains from around the world to determine whether its presence is correlated with an enhanced resistance phenotype. The exact role of this substitution in conferring pyrethroid insensitivity requires direct electrophysiological analysis of isolated channels containing this substitution with and without the leucine to phenylalanine change, e.g., by site-directed mutagenesis and *in vitro* expression of the cloned sequences.

Resistance factors for pyrethroids recorded in this study were considerably higher than those reported to be conferred by *kdr* alleles in the house fly (26) and the African bollworm, *H. armigera* (14). However, levels of nerve insensitivity to pyrethroids were also higher than the ones reported for *H. armigera* (52) and from previous work with *P. xylostella* (11). Although we cannot exclude the possibility that increased metabolism also contributes to pyrethroid resistance in the FEN strain, the concordance between bioassay and electrophysiological data, combined with results of synergism studies, genetic crosses, and molecular analyses, supports a primary role for a *kdr* mechanism, possibly conferred by a more potent allele than those described to date. Given its severe practical implications, the incidence and dynamics of this mechanism deserve more detailed investigation.

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