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Why are there so few resistance-associated mutations in insecticide target genes?

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The genes encoding the three major targets of conventional insecticides are: *Rdl*, which encodes a γ-aminobutyric acid receptor subunit (RDL); *para*, which encodes a voltage-gated sodium channel (PARA); and *Ace*, which encodes insect acetylcholinesterase (AChE). Interestingly, despite the complexity of the encoded receptors or enzymes, very few amino acid residues are replaced in different resistant insects: one within RDL, two within PARA and three or more within AChE. Here we examine the possible reasons underlying this extreme conservation by looking at the aspects of receptor and/or enzyme function that may constrain replacements to such a limited number of residues.

Keywords: acetylcholinesterase; *Rdl*; γ-aminobutyric acid receptor; *para*; voltage-gated sodium channel; insecticide resistance

1. INTRODUCTION

The three major targets of conventional insecticides are: (i) the γ-aminobutyric acid (GABA) receptor containing RDL subunits encoded by the gene *Resistance to dieldrin* or *Rdl*, the target for cyclodiene insecticides and the recently introduced fipronils; (ii) the PARA voltage-gated sodium channel encoded by the gene *para*, the target site for DDT and pyrethroids; and (iii) insect acetylcholinesterase (AChE) encoded by the gene *Ace*, the target site for organophosphorus (OP) and carbamate insecticides. Genes encoding all three of these target sites have been cloned from *Drosophila melanogaster*, supporting the previously proposed role of this insect as a model in which to study insecticide resistance (Wilson 1988; ffrench-Constant *et al.* 1992). However, homologues of these *Drosophila* target-site genes have recently also been cloned from a range of pest insects and the underlying resistance-associated mutations compared. Interestingly, despite the wide range of insects studied, and presumed differences in the modes of insecticide selection, the same residues are consistently replaced in the same receptors and/or enzymes: a single residue in RDL, two residues in PARA and three or more in AChE.

The purpose of the current paper is to examine the question: why are there so few resistance-associated mutations in the genes encoding these receptors and/or enzymes? This will be achieved by examining our work on receptor and/or enzyme function in relation to the likely functional constraints placed on these important components of the insect nervous system. In each case a working model or hypothesis is presented to account for the conservation of amino-acid replacements observed in the context of what we know about the normal function of the receptor and which residues may interact with the insecticide.

2. CONSERVATION OF RESISTANCE-ASSOCIATED MUTATIONS

(a) *The* Rdl-*encoded GABA receptor*

The gene *Rdl*, encoding the GABA receptor subunit RDL, was cloned from a *D. melanogaster* mutant resistant to cyclodiene insecticides and picrotoxinin (PTX), a vertebrate $GABA_A$ receptor antagonist (ffrench-Constant *et al.* 1991). The pharmacology of RDL-containing insect receptors and the relationship of these GABA receptors to those found in vertebrates has recently been reviewed elsewhere (Hosie *et al.* 1997). To determine the resistance-associated mutation(s), we examined worldwide collections of resistant *D. melanogaster*. In these strains we consistently documented the replacement of the same amino acid, alanine302 with serine (ffrench-Constant *et al.* 1993*b*). Following functional expression of RDL subunits as homomultimers in a range of heterologous expression systems (ffrench-Constant *et al.* 1993*a*; Lee *et al.* 1993), we also confirmed the functional relevance of this mutation by showing that replacement of alanine302 with serine results in insensitivity of the resulting GABA-gated chloride channels (ffrench-Constant *et al.* 1993*a*). Importantly, RDL-containing GABA receptor subunits are widely expressed in the insect nervous system (Aronstein & ffrench-Constant 1995; Aronstein *et al.* 1996); despite the fact that we can document that RDL co-assembles with another unidentified subunit (via observed differences of channel conductance *in vivo* and *in vitro*) (Zhang *et al.* 1995), RDL expression *in vitro* reconstitutes most of the pharmacology observed in insect GABA receptors *in vivo* (ffrench-Constant *et al.* 1993*a*; Zhang *et al.* 1994, 1995). A survey of a wide range of other insect species, including several beetles, a mosquito (*Aedes aegypti*), the whitefly *Bemisia tabaci*, and a cockroach (*Blattella germanica*) (Thompson *et al.* 1993; ffrench-Constant *et al.* 1994;

Anthony *et al.* 1995*b*), showed that this alanine-to-serine replacement was the most common resistance-associated replacement. More rarely, in a different fruit fly, *D. simulans* (ffrench-Constant *et al.* 1993*b*), and in the aphid, *Myzus persicae* (N. Anthony and R. ffrench-Constant, unpublished data), the same residue is replaced with a glycine.

(b) *The PARA voltage-gated sodium channel*

The gene *para*, which encodes the PARA voltage-gated sodium channel, was cloned from *Drosophila* in the laboratory of Barry Ganetzky on the basis of the *temperature sensitive* paralytic phenotypes displayed by $para^{ts}$ alleles (Loughney *et al.* 1989). The PARA sodium channel appears to be the major sodium channel in insects and is a large polypeptide composed of four homology domains (I–IV), each containing six proposed hydrophobic membrane-spanning domains (S1–S6) (Loughney *et al.* 1989). After linkage studies that correlated resistance to DDT and pyrethroids with the location of the *para*-homologous sodium-channel gene in *knockdown resistant* (*kdr*) house flies (Williamson *et al.* 1993; Knipple *et al.* 1994) and cockroaches (Dong & Scott 1994), *para* homologues were cloned from both species and the underlying resistance-associated mutations examined (Miyazaki *et al.* 1996; Williamson *et al.* 1996; Dong 1997). Again, amino-acid replacements were confined to only two positions. The first, associated with the original *kdr* strain, was in the S6 hydrophobic segment of homology domain II (termed IIS6). The second, associated with another more resistant allele, termed *super-kdr*, was found as a double mutant with both the *kdr* mutation and a second replacement in the intracellular loop between IIS4 and IIS5. The '*kdr*-like' replacement is similar in the housefly (Miyazaki *et al.* 1996; Williamson *et al.* 1996), the horn fly (Guerrero *et al.* 1997), the cockroach (Dong 1997), the tobacco budworm *Heliothis virescens* (Park & Taylor 1997), the aphid *M. persicae* (Field *et al.* 1997), and in the mosquito *Anopheles gambiae* (Martinez-Torres *et al.* 1998), whereas the second *super-kdr* type mutation has only been found in resistant houseflies (Williamson *et al.* 1996) and horn flies (Guerrero *et al.* 1997). Interestingly, so far these replacements associated with field-collected resistance alleles have been found to reside within the second homology domain of the protein.

Recently, the housefly *para* homologue has been functionally expressed in *Xenopus* oocytes and the leucine1014 to phenylalanine replacement (*kdr* mutation) has been shown to confer ten-fold insensitivity to cismethrin on the resulting sodium channels (Smith *et al.* 1997).

(c) *The acetylcholinesterase-encoding locus,* Ace

AChE degrades the neurotransmitter acetylcholine in the insect synapse and is the primary target site for OP and carbamate insecticides, which inhibit the enzyme. The gene *Ace*, which encodes insect acetylcholinesterase (AChE), was again cloned from *Drosophila* based on the knowledge of its location via isolation of a range of different mutants (Hall & Spierer 1986). The screening of a range of different *D. melanogaster* and housefly strains has identified a range of different amino-acid replacements putatively causing resistance (reviewed by Feyereisen (1995)). The functional significance of several of these have been tested in the *Drosophila* enzyme following the heterologous expression of different mutants in *Xenopus* oocytes and direct testing of their insensitivity to a range of different insecticide inhibitors (Mutero *et al.* 1994). A comparison of the different putative resistance-associated amino-acid replacements in the fruit fly and the housefly (Feyereisen 1995) again suggests that only a limited subset of replacements cause resistance despite the widely differing sizes and structures of different OP and carbamate insecticides. However, many of these resistance-associated residues are predicted to lie within or close to the active-site gorge of this enzyme, based on superimposition of the insect amino-acid sequence on the three-dimensional crystal structure obtained from *Torpedo* (Mutero *et al.* 1994). The role of other replacements linked to AChE insensitivity in other insects, such as the Colorado potato beetle *Leptinotarsa decemlineata* (Zhu & Clark 1997), remains to be functionally proven.

3. IMPLICATIONS FOR RECEPTOR AND ENZYME STRUCTURE AND FUNCTION

(a) *The RDL containing GABA receptor*

RDL GABA receptor subunits co-assemble in a variety of expression systems to give functional homomultimeric GABA-gated chloride-ion channels (ffrench-Constant *et al.* 1993*a*; Lee *et al.* 1993). Further, despite the fact that the conductance of these channels differs from those of RDL-containing channels in the insect nervous system (Zhang *et al.* 1994, 1995) (showing that other subunits are present in RDL-containing receptors in the insect nervous system), these homomultimers reconstitute much of the GABA receptor pharmacology displayed *in vivo*, notably PTX and cyclodiene sensitivity and bicuculline insensitivity (Zhang *et al.* 1995). The observation that the replacement of alanine302 with serine confers insecticide insensitivity leads to the simplest hypothesis, that PTX and cyclodienes actually bind within the ion-channel pore (as alanine302 is within the second membrane-spanning region, which is predicted to line the chloride ion-channel pore by homology with structural and modelling work performed in the closely related nicotinic acetylcholine receptor (Leonard *et al.* 1988)). However, our consistent finding of replacements of this same amino acid (with either a serine or a glycine) in a wide range of different insects leads us to examine why only this residue is replaced in resistance. Thus, for example, the binding site of PTX and cyclodienes is unlikely to interact with only this single residue, therefore why can other residues in the binding site not be replaced to give resistance?

To examine this question we performed a biophysical analysis of wild-type and mutant RDL receptors via the patch clamping of cultured *Drosophila* neurons (Zhang *et al.* 1994). Detailed analysis revealed significant but small shifts in a range of channel parameters, namely small changes in the shape of the GABA dose–response curves, small changes in both the inward and the outward conductance of the channel, and a net stabilization of the channel's open state. However, the largest change in a single channel parameter was in the rate of receptor desensitization (Zhang *et al.* 1994; ffrench-Constant *et al.* 1995). This can be described as the rate at which the channel returns to its normal activatable state under prolonged exposure to GABA (figure 1*a*). Thus, channels

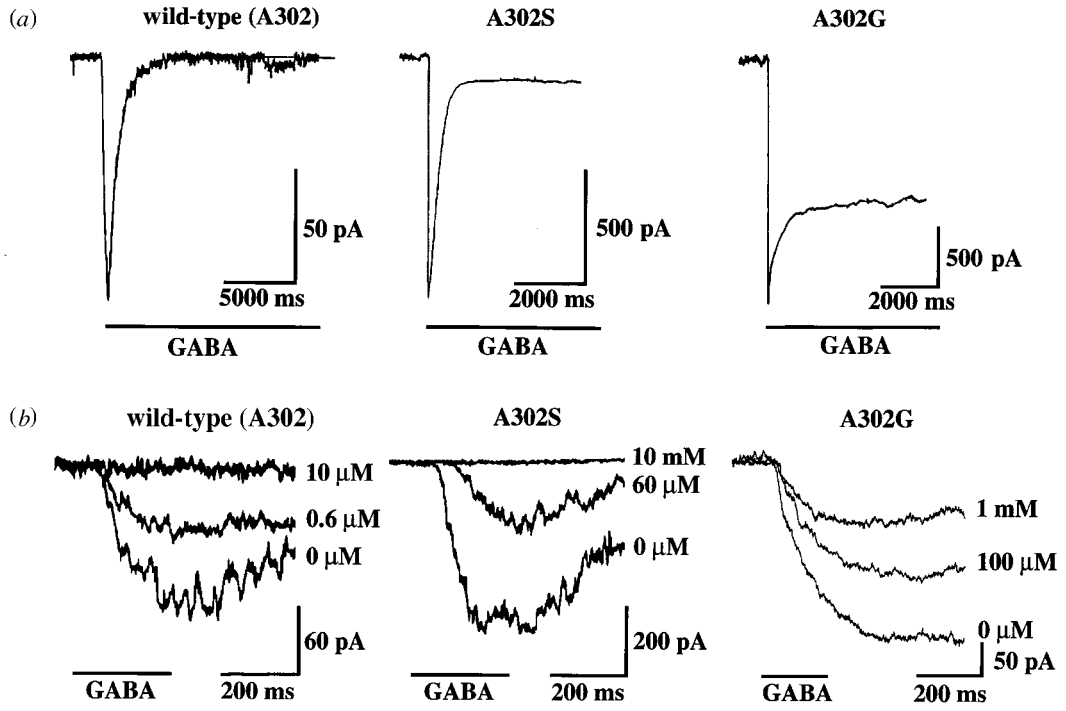

Figure 1. Decreased rates of GABA receptor desensitization are correlated with increased levels of PTX resistance in two different *Rdl* mutants. (*a*) Rates of receptor densitization in wild-type (alanine302) neurons and in those from two different resistant mutants: alanine302 to serine (A302S) and alanine302 to glycine (A302G). (*b*) Levels of PTX resistance observed in the same mutants. Note that wild-type receptors can be blocked by 10 μM PTX whereas receptors containing alanine302 to serine require 10 mM and those containing alanine302 to glycine also require more than 1 mM for partial block.

containing alanine302 to serine replacements desensitized 29 times more slowly than wild-type ones and desensitization was incomplete (figure 1*a*) Although at first these changes are difficult to reconcile with the interaction of the receptor with the antagonist, other workers have proposed that PTX binds preferentially to the desensitized state of the vertebrate $GABA_A$ receptor (Newland & Cull-Candy 1992). We have therefore formulated a working model whereby replacements of alanine302 have a dual role in both: (i) interacting directly with the drug binding site within the ion-channel pore, and (ii) allosterically destabilizing the insecticide's preferred desensitized state of the RDL-containing GABA receptor (Zhang *et al*. 1994; ffrench-Constant *et al*. 1995).

Our recent experiments on cultured *Drosophila* neurons have added weight to this hypothesis by demonstrating that the replacement of alanine302 with a glycine residue (the alternative resistance-associated replacement found in nature) also confers resistance to PTX and is again (like alanine302 to serine) associated with slower and incomplete receptor densensitization (compare figure 1*a* and 1*b*). Further experiments have been performed to test a corollary of this hypothesis: that replacements of other residues known to affect rates of receptor desensitization should also affect resistance but to a lesser extent. In conclusion, current data are consistent with a model whereby replacements of alanine302 cause resistance by interacting directly with the insecticide binding site and also allosterically by destabilizing the insecticide's preferred (desensitized) conformation of the receptor. This implies that replacements having effects on only one process or the other would not confer sufficient resistance on the insect for insecticide selection to be effective in the field.

(b) *The PARA voltage-gated sodium channel*

Owing to the relative ease of isolating large numbers of *para* mutants based on their *temperature sensitive* (*ts*) phenotypes, we screened an existing collection of *ts* mutants to indentify any that were also resistant to DDT and pyrethroid insecticides (Pittendrigh *et al*. 1997). This approach has the advantage of using temperature sensitivity as an independent screen for replacements that affect insecticide sensitivity of the channel, and may therefore identify residues that interact with insecticides but may carry sufficient fitness costs in the whole insect to prevent their selection in natural populations. This technique can therefore potentially identify a larger range of residues affecting the interaction of the channel with insecticides than those found in nature.

We examined DDT resistance in a group of 12 independent *para* mutants, including two that cause dominant temperature-sensitive paralysis, nine showing recessive temperature sensitivity and one that causes a smell-blind phenotype (Pittendrigh *et al*. 1997). Of these 12 mutants, we found that six were also associated with DDT insensitivity. As expected, resistance was sex-linked, as *para* is X-linked in *D. melanogaster*, and not responsive to the synergist piperonyl butoxide (PBO), which suppresses metabolic resistance (Pittendrigh *et al*. 1997). Two general aspects of the overall nature of these mutations are noteworthy. First, exactly half of the temperature-sensitive mutations examined conferred resistance, despite their not being preselected with insecticides. This observation may imply that only a limited number of replacements can be generated in the sodium channel polypeptide which confer a temperature-sensitive and/or resistant phenotype. Second, although many of these mutations

occupy positions equivalent to the *kdr* and *super-kdr* mutations (figure 2), none reside in domain II. This might imply that replacements in homology domain II are less prone to confer temperature-sensitive phenotypes and this avoidance of a potentially adverse fitness cost may be part of the reason why most resistance-associated replacements appear confined to this domain in natural populations. However, alternatively, we note that a further '*kdr*-like' replacement (i.e. located in the equivalent section of S6) correlated with resistance in *Heliothis virescens* lies in homology domain I and not domain II (Park & Taylor 1997). This observation suggests that resistance-associated replacements can be found in other homology domains and that their previous documentation solely in domain II may just be an artefact of (i) the limited number of species examined, and (ii) the emphasis on re-examining the same region, i.e. II S6.

Several specific aspects of the relative locations of the different replacements in *D. melanogaster* are also interesting (Pittendrigh *et al*. 1997). Thus the replacements fall into three categories (figure 2): (i) those that are '*kdr*-like', such as $para^{74}$, which resides in an equivalent position in S6 but in the third rather than the second homology domain; (ii) those that are '*super-kdr*-like', such as $para^{D,N7}$ and $para^{ts1}/para^{ts2}$ (independent occurrences of the same point mutation), which lie within the intracellular S4–S5 linker but in domains III and I, respectively; and (iii) those that occupy a novel position, such as $para^{DTS2}$/$para^{D,N43}$ (again independent examples of the same mutation), which lie within the S5–S6 loop that may actually form part of the sodium-channel lining. In terms of resistance levels generated, independently, the '*kdr*-like' or '*super-kdr*-like' replacements in *D. melanogaster para* both confer low levels of resistance to DDT. Interestingly, and similarly to *super-kdr* in the housefly, combination of a '*kdr*-like' and '*super-kdr*-like' mutation in *Drosophila* heterozygous for the two alleles also gives increased resistance to the type II (cyano-group-containing) pyrethroids in the whole fly.

Again, the simplest interpretation of these data is that these replacements all denote the binding site for either DDT and type I ('*kdr*-like' replacements) or additionally type II ('*super-kdr*-like' replacements) pyrethroids (Williamson *et al*. 1996). However, our results suggest the possibility of a different mechanism. The identification of DDT-resistant mutations at sites nearly equivalent to *kdr* and *super-kdr* but in other homology domains suggests that if these mutations do indeed define a binding site, it is likely to be composed of residues contributed by each of the four homology domains. This possibility seems reasonable given the essentially tetrameric structure of the sodium-channel α-subunit. However, it is also possible that the sodium-channel mutations characterized here, and in previous studies, confer resistance to pyrethroids and DDT by a mechanism other than the direct alteration of the binding site. The pharmacological effect of these insecticides is to cause persistent activation of sodium channels by delaying the normal voltage-dependent mechanism of inactivation (Soderlund & Bloomquist 1989). Rather than directly affecting the binding of pyrethroids and DDT, the mutations may confer resistance by causing functional changes in sodium-channel properties that compensate for or that alleviate the consequences of the insecticide. For example, if the mutations altered the voltage dependence or kinetics of activation or inactivation, the neurotoxic effects of the insecticide could be reduced or overridden. In this context we note that recent functional analysis of the equivalent of the *kdr*-associated amino-acid replacement in a sodium channel in the rat has provided evidence for accompanying changes in voltage dependence (Vais *et al*. 1997).

This interpretation is particularly consistent with the resistant mutations that map to the S4–S5 loop in the different homology domains, because a variety of evidence from various systems suggests the importance of this region in channel function. Mutations in this loop are known to reduce fast inactivation of potassium channels (Isacoff *et al*. 1991). Furthermore, mutations in mammalian sodium channels at or near the site defined by the $para^{D,N7}$ mutation are associated with a variety of abnormalities. One form of long QT syndrome, an inherited cardiac arrhythmia, is caused by an amino-acid substitution in the SCN5A channel at a site adjacent to the residue affected by $para^{D,N7}$ (Wang *et al*. 1995). At the same residue as $para^{D,N7}$, an alanine-to-threonine replacement in the human SCN4A channel causes paramyotonia congenita, a disorder associated with decreased kinetics of sodium-channel inactivation (McClatchey *et al*. 1992; Yang *et al*. 1994). The same replacement in a mouse neuronal sodium channel produces the *jolting* phenotype (Kohrman *et al*. 1996). Functional studies indicate that this mutation significantly shifts the voltage dependence of activation in the depolarizing direction. The $para^{ts1}$ mutation resides at the identical location in homology domain I and may cause functional perturbations analogous to those caused by mutations in the vicinity of $para^{D,N7}$. The $kdr/para^{74}$ mutations in S6 transmembrane segments might also alter the gating properties of sodium channels in some distinctive manner.

Our finding of synergistic resistance to pyrethroids in $para^{74}/para^{D,N7}$ heterozygotes is of particular interest. The changes conferred by these two mutations in homology domain III are in locations analogous to those of the two mutant sites in homology domain II in *super-kdr* strains of houseflies; the elevated levels of resistance in the heterozygote approximate the enhanced resistance in *kdr* compared with *super-kdr* strains. However, the two circumstances are very different. In *super-kdr* mutants, the two lesions reside in the same polypeptide (i.e. they are in *cis*); in $para^{74}/para^{D,N7}$ heterozygotes, the lesions reside in different polypeptides (i.e. in *trans*). The increased resistance in *super-kdr* housefly strains is presumed to reflect a combined deficit in pyrethroid binding caused by the doubly mutant polypeptide. This explanation seems inadequate to account for the phenotype of $para^{74}/para^{D,N7}$ heterozygotes. However, this phenotype can be reconciled with the alternative view that resistance is mediated via functional alterations of the encoded sodium-channel polypeptides. The particular functional defect associated with one mutation could enhance the perturbation caused by the other. For example, one mutation could alter the inactivation mechanism, leading to a slight depolarization of membrane potential and the other could alter the voltage dependence of inactivation. The combined effect in double heterozygotes could be very different from the effect of either mutation alone or when heterozygous with

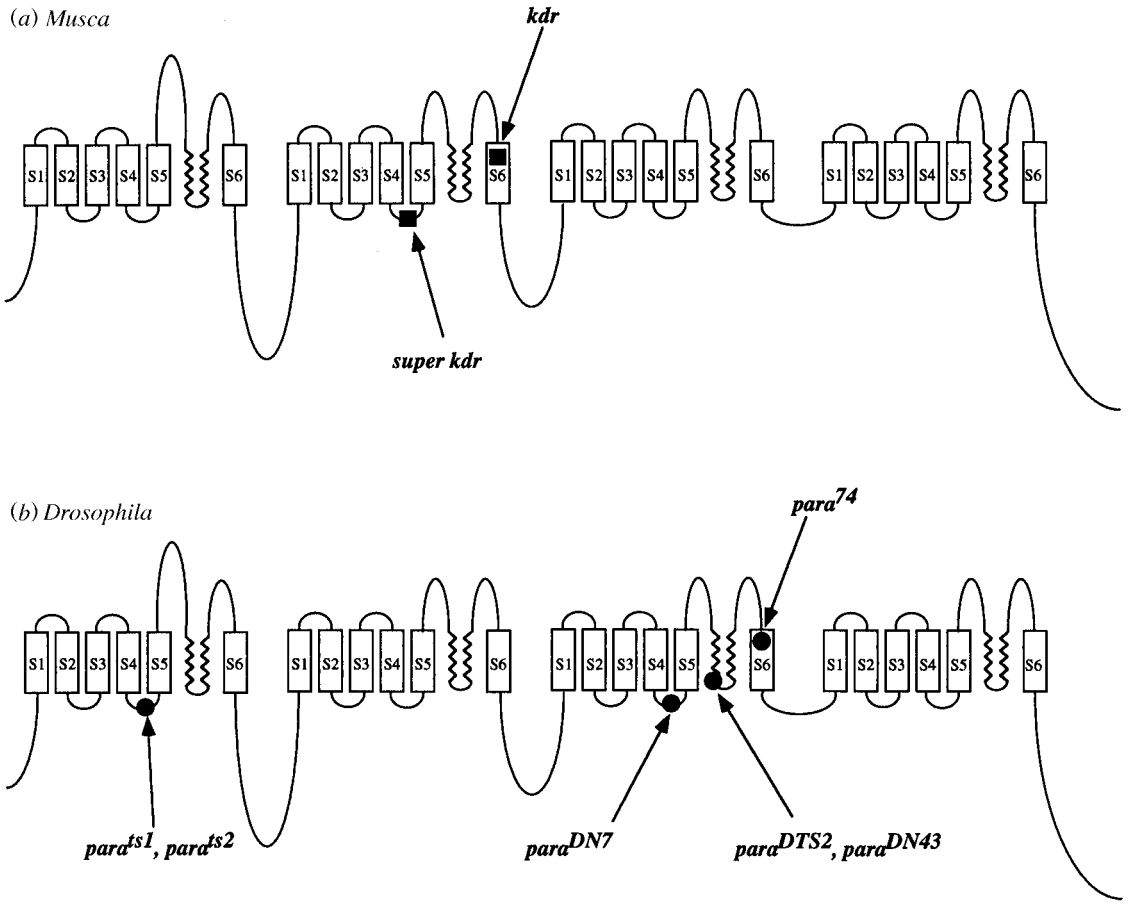

Figure 2. Comparison of the relative locations of DDT/pyrethroid resistance-associated replacements in the *para*-encoded voltage-gated sodium channel of *Drosophila melanogaster* and those in the house fly *Musca domestica*. (*a*) The location of the replacements associated with *kdr* and *super-kdr* in the *para*-homologous channel from *Musca*. Note that these are both in homology domain II. (*b*) The location of replacements in *Drosophila para*, which were isolated on the basis of temperature-sensitive paralytic phenotypes but which also confer DDT/pyrethroid resistance. Two classes of these mutations are in positions approximately equivalent to those in *kdr* and *super-kdr*; the position replaced in both $para^{DTS2}$ and $para^{DN43}$ represents a novel third class of resistance-associated mutation. Note, however, that none of the temperature-sensitive replacements resides in domain II.

a wild-type allele. This interpretation is also consistent with the observed phenotypic dominance of the double heterozygote in the presence of an extra copy of the *para* locus encoding wild-type sodium-channel subunits. This situation is analogous to the dominant effect of mutations causing hyperkalaemic periodic paralysis (Ptacek *et al.* 1991; Rojas *et al.* 1991; McClatchey *et al.* 1992) in humans, where the misbehaviour of a small percentage of mutant sodium channels at the resting membrane potential shifts all of the other channels (including wild-type channels) to an inactivated state (Cannon *et al.* 1991).

We are currently performing a direct electrophysiological analysis of the resistant *Drosophila para* mutants to confirm their importance in defining resistance-associated replacements and also to separate the relative roles of the different mutations in altering channel function and/or insecticide binding. To date, our results support a hypothesis whereby some replacements interact with channel inactivation. Thus, for example, the application of 500 nM allethrin to wild-type channels removed inactivation and prolonged tail currents whereas it had little or no effect on $para^{74}$ mutant channels (R. Martin, B. Pittendrigh, J. Liu, R. Reenan, B. Ganetzky and D. Hanck, unpublished data). However, we will need to examine '*kdr*-like' and '*super-kdr*-like' mutations independently and together in the same fly (in *trans* rather than in *cis*) to test any potential dual effect on channel function and insecticide insensitivity.

(c) *Acetylcholinesterase*

To date, the functional relevance of potential resistance-associated replacements in insect AChE has only been tested in *D. melanogaster* (Mutero *et al.* 1994). Therefore, to confirm the relevance of putative resistance-associated mutations in a pest insect we have chosen the *Ace* gene from the yellow fever mosquito, *A. aegypti*, as a model system in which to study the effects of mutations on a pest insect enzyme (Anthony *et al.* 1995*a*; Vaughan *et al.* 1997). This mosquito is a vector of both yellow fever and dengue; we have recently found insensitive AChE in populations of this species in Trinidad (Vaughan *et al.* 1998).

Acetylcholinesterase genes (*Ace* homologues) have now also been cloned from a number of insect species including the mosquitoes *Anopheles stephensi* (Hall & Malcolm 1991) and *Culex pipiens* (C. Malcolm, unpublished data). However, although insecticide-insensitive AChE has been widely documented biochemically from a range of both *Culex* and *Anopheles* mosquitoes (Ayad & Georghiou 1975; Hemingway & Georghiou 1983; Hemingway *et al.* 1985; Raymond *et al.* 1986; Haas *et al.*

Table 1. *Residues replaced in* Aedes aegypti *AChE compared with those found in other insects with insensitive AChE*

(Only the replacements in *Drosophila* have been tested by site-directed mutagenesis and functional expression. Note that the mutations made in the *Aedes* enzyme correspond to those found in other insects and not to any yet found in natural populations of *A. aegypti*.)

		numbering in AChE of *Torpedo californica*						
insect	strain	78	129	151	227	238	288	328
Drosophila melanogaster	Saltillo	Phe > Ser	Ile > Val	—	Gly > Ala	—	Phe > Tyr	—
	Bygdea	—	Ile > Val	—	Gly > Ala	—	—	—
	Pierrefeu	—	Ile > Thr	—	Gly > Ala	—	—	—
	MH19	—	—	—	—	—	Phe > Tyr	—
Musca domestica	77M	—	—	Val > Leu	Gly > Ala	—	Phe > Tyr	—
	CH2	—	—	—	Gly > Ala	—	Phe > Tyr	—
	49R	—	—	—	—	—	—	Gly > Ala
Leptinotarsa decemlineata	AZ-R	—	—	—	—	Ser > Gly	—	—
mutagenized *Aedes aegypti*	—	Phe > Ser	—	—	Gly > Ala	—	Phe > Tyr	—

1988; ffrench-Constant & Bonning 1989; Tang & Cammak 1990), no resistance-associated mutations have been described from mosquitoes themselves.

Analysis of insecticide-insensitive AChE in mosquitoes has also recently been complicated by the discovery of two AChEs (AChE1 and AChE2) in *Culex pipiens* (Bourguet *et al*. 1996*b*). These differ in their sensitivity to insecticide inhibition. In susceptible insects, AChE1 can be inhibited by a fixed dose of a carbamate insecticide (5×10^{-4} M propoxur) whereas AChE2 is unaffected by this concentration (Bourguet *et al*. 1996*a*). Linkage mapping of the *Ace* gene of *Culex pipiens* suggests that it is at a sex-linked locus (C. Malcolm, personal communication), whereas resistance in *Culex* is not sex-linked. However, detailed analysis of inhibition profiles in a range of other mosquito species, including *Aedes aegypti*, suggests that most other mosquitoes bear only a single *Ace* locus and that *C. pipiens* may therefore be an exception. Interestingly, linkage mapping of the *A. aegypti* clone discussed here shows that it maps extremely close to the sex-determining locus (Severson *et al*. 1997); this result suggests that resistance should be sex-linked.

We cloned a section of the *Ace* locus from *A. aegypti* with the use of degenerate primers in the polymerase chain reaction and then isolated a full-length clone from an adult cDNA library (Anthony *et al*. 1995*a*). Functional expression was achieved in baculovirus-infected SF21 insect cells (Anthony *et al*. 1995*a*) and the effects of the resistance-associated amino-acid replacements found in *Drosophila* and the housefly were tested by mutagenesis of the *Aedes* gene *in vitro* (table 1). Resistance of AChE to OP and carbamate insecticides is due to modifications of the active site (amino-acid replacements) and these modifications also appear to alter the catalytic activity of the enzyme towards insecticides and substrates. Therefore, the mutant forms of *Aedes* AChE all behaved differently from the wild-type when initial rates of activity were assayed with acetyl, propionyl and butyryl thiocholine iodides (ASCI, PSCI and BSCI). In most cases, the initial rate was highest with ASCI, followed by PSCI and then BSCI. However, in the single mutant F350Y and the double mutant F105S+F350Y, higher rates of activity with PSCI and BSCI than with ASCI were observed, for which the activity was approximately 20% of that of the wild-type enzyme (Vaughan *et al*. 1997). In *D. melanogaster*, the corresponding F368Y mutant also had higher rates of activity with PSCI and BSCI (Fournier *et al*. 1996). Furthermore, decreased rates of reaction with ASCI for insensitive forms of AChE have been documented in both *Anopheles albimanus* and *C. pipiens* (ffrench-Constant & Bonning 1989; Bourguet *et al*. 1996*a*). It will therefore be interesting to investigate, when rates of reaction with ASCI fall, whether replacements equivalent to F368Yare found.

In terms of resistance, mutagenesis of *D. melanogaster Ace* showed a correlation between the number of mutations in the expressed enzyme and the bimolecular constant (k_i) ratio of the mutated compared with the wild-type from of the enzyme (Mutero *et al*. 1994). In general, the greater the number of resistance-associated amino-acid replacements, the higher the resistance ratio, although this was not always the case. In the mutagenized *Aedes* enzyme, a similar pattern of relative insensitivity was found (figure 3). However, the comparison is complicated by the presence of one of the mutations in the wild-type form of the *A. aegypti Ace*: the Ile199Val mutation in *D. melanogaster* AChE is already present in *A. aegypti* AChE (Val185). Whereas in *D. melanogaster* none of the single point mutations gave rise to significant levels of resistance, the G285A mutant gives more than a 20-fold increase in the resistance ratio with the OP paraoxon. For both classes of insecticides, the double mutant G285Y+F350Y and the triple mutant gave substantial increases in the resistance ratio with all the insecticides tested (figure 3).

The three-dimensional structure of *Torpedo californica* AChE has been determined (Sussman *et al*. 1991), and it is thus possible to superimpose other AChEs on this. G303 in *D. melanogaster* (G285 in *A. aegypti*) is thought to affect the orientation of the active-site serine, which is phosphorylated by OPs and carbamylated by carbamates; F368 (F350 in *A. aegypti*) is near the acyl moiety of the bound substrate (Mutero *et al*. 1994). The presence of both these mutations in *A. aegypti* AChE has a profound effect on the binding of insecticide, resulting in the high insensitivity ratios observed.

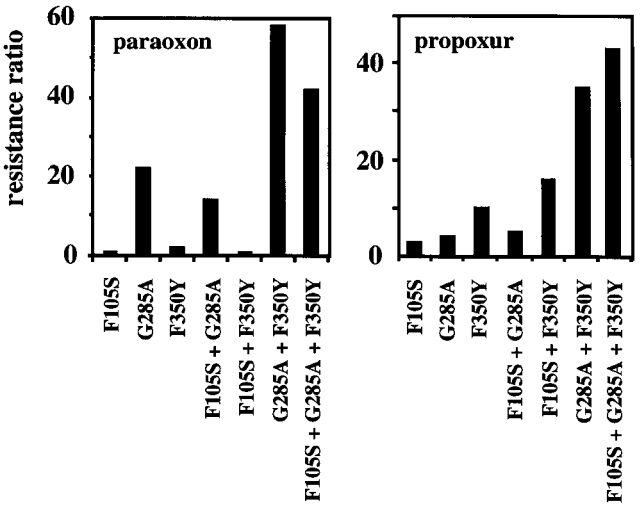

Figure 3. Site-directed mutagenesis of the mosquito *Ace* gene confers insecticide insensitivity on baculovirus-expressed AChE mutant enzymes. Levels of AChE insensitivity to inhibition by paraoxon (an OP) and propoxur (a carbamate). Histograms show the effects on the bimolecular velocity constant (k_i) of single and combined replacements (single-letter code). The ordinate corresponds to the ratio of the resistant to the susceptible k_i, here termed the insensitivity ratio.

These studies have demonstrated that the same mutations that cause insecticide resistance in *D. melanogaster* AChE can also confer insensitivity to the enzyme of the pest mosquito *A. aegypti* when its gene is mutagenized. Given the similarity of the predicted amino-acid sequence of the *Aedes* enzyme with the resistance-associated enzyme from *Drosophila*, the present study forms a useful model for examination of mosquito *Ace* homologues. The ease of expression of the *A. aegypti* AChE enzymes in the baculovirus system should permit a thorough kinetic analysis of enzyme inhibition similar to that performed for the *Drosophila* enzyme. This should give an explanation of how the amino-acid replacements within the resistance-associated AChE mutants affect the binding of insecticide to the enzyme and provide a detailed understanding of their affects on substrate specificity and target-site resistance.

4. CONCLUSIONS

Despite the potential complexity (e.g. the predicted multisubunit nature of RDL-containing GABA receptors), and/or large size (e.g. the PARA voltage-gated sodium channel) of the receptors and enzymes that constitute the primary targets for conventional insecticides, the resistance-associated amino-acid replacements documented to date are strikingly few. In this paper, we have attempted to reconcile this extreme conservation with working hypotheses on the role of these replacements in receptor and/or enzyme insecticide insensitivity. This was achieved by detailed functional analysis of the insect GABA receptor and also by a mutagenesis of the insect voltage-gated sodium channel that relies on a phenotype independent of resistance, namely temperature-sensitive paralysis. This latter mutagenesis underscores the potential importance of temperature-sensitive phenotypes in isolating toxicologically relevant mutations in insects and in this respect it is also interesting to note with hindsight that $Rdl^{alanine\,302>serine}$, originally isolated on the basis of insecticide resistance, is also itself a temperature-sensitive paralytic mutant (ffrench-Constant *et al.* 1993*c*).

To explain this striking degree of conservation in replacements in both the GABA-gated chloride channel and potentially the voltage-gated sodium channel, we suggest that the interaction of more than one channel function with insecticide binding sites may be important in constraining the location of resistance-associated replacements. Thus, in the RDL-containing GABA receptor, alanine302 interacts both directly with the insecticide binding site and also allosterically by destabilizing the insecticide's preferred desensitized state of the receptor. In the *para* voltage-gated sodium channel, the observation that individual mutations in different channel polypeptides can combine in *Drosophila* to cause effects similar to those of mutations found in the same polypeptide, also suggests that each mutation may have a unique role in affecting channel function. For example, one replacement could alter the inactivation mechanism, leading to a slight depolarization of the membrane, and the second could alter the voltage dependence of inactivation. In AChE we need to test the functional relevance of other resistance-associated mutations found in pest insects to further test the hypotheses advanced for replacement insecticide interactions in the *Drosophila* enzyme (Mutero *et al.* 1994).

Such studies of the detailed biophysics and biochemistry of the receptors and enzymes associated with insecticide targets not only elucidate potential binding sites but also illustrate that these proteins are dynamic molecules that interact in various conformations with their antagonists and agonists. The effect of resistance-associated replacements may therefore not be easily mimicked by static models based on simple 'lock-and-key' type binding interactions. Although currently confined to the three historically important targets, such studies will also become important in the face of likely insensitivity in new targets such as the nicotinic acetylcholine receptor (nACh), the target for important new compounds such as immidacloprid and spinosad. In the latter case, the close structural relationship between GABA receptors and nACh receptors may forewarn us of potential resistance mechanisms to these highly effective compounds.

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