Interaction between Acetylcholinesterase and Choline Acetyltransferase: an Hypothesis to Explain Unusual Toxicological Responses

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Abstract: Organophosphorus and carbamate insecticides are thought to have only one target site, acetylcholinesterase (EC 3.1.1.7). When this enzyme is inhibited, the neurotransmitter acetylcholine is not metabolized and polarization of the post-synaptic membrane does not take place. But, what happens when the cholinesterase becomes resistant or when neurotransmitter levels are diminished? Here, we report results suggesting that choline acetyltransferase (EC 2.3.1.6), the enzyme responsible for the acetylcholine production, now be involved either as an alternative pesticide target site or as a factor enhancing survival during insecticide exposure. This underlines the concept that the pivotal step for insecticide toxicity is not the acetylcholinesterase activity but the amount of acetylcholine present. This latter can only fluctuate between as upper and a lower threshold, and crossing one of these two thresholds leads to the death of the insect. The interaction between acetylcholinesterase and choline acetyltransferase activities would explain the astonishing toxicological phenomena that, in some conditions, mortality decreases when insecticide concentration increases.

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1 INTRODUCTION

Acetylcholinesterase (ACHE; acetylcholine acetyl-
hydrolase, EC 3.1.1.7) and choline acetyltransferase
(CHAT, acetyl-CoA-Choline-O-acetyltransferase, EC
2.3.1.6) are two key enzymes in the cholinergic system
because they regulate the level of acetylcholine (ACh),
the primary sensory neurotransmitter in arthropods. 1,2
CHAT is implicated as a regulatory step for ACh pro-
duction1 whereas AChE terminates nerve impulses by
catalysing the hydrolysis of ACh. Of the two enzymes,
only AChE has been considered for the develop-
ment of inhibitors as insecticides. Thus, organophosphorus
and carbamoyl insecticides have properties analogous to ACh
but are termino-inhibitors: they quasi-irreversibly inhibit ACHE by phos-
phorylating or carbamoylating the active-site serine. 4
ACHE inhibition leads to an accumulation of ACh in
the synapses which, in turn, leaves the ACh receptors
permanently open, resulting in the death of the insect. 4,5
Insecticide resistance would arise through the selec-
tion of any mechanisms which balance ACh accumula-
tion. One of them is an increase in ACh degradation: it
corresponds either to an overproduction of the enzyme
or to a decrease of AChE inhibition due to the appear-
ance and selection of altered ACHEs less sensitive to the
insecticide.6 Overproduction has not been studied in
resistant populations due to the difficulty in estimating
the AChE content in insects. However, modification of
ACHE amounts in Drosophila has been correlated to
insecticide sensitivity. 7 Concerning ACHE modification,
some point mutations have been reported in the fruit
fly, 8,9 housefly (Williamson and Devonshire, pers.
comm.) and Colorado potato beetle. 10
A second resistance mechanism would be a decrease
in ACh production because of a reduced CHAT activity.
Surprisingly, this second resistance mechanism has not
yet been reported.
In this paper we report results suggesting the influ-
ence of modifications of CHAT activity on pesticide
resistance, showing how CHAT modification may
provide insecticide resistance, and that CHAT could be
a pesticide target-site or a modifier of pesticide resis-
tance.

2 EXPERIMENTAL METHODS

2.1 Mosquito strains

Three strains of the mosquito Culex pipiens L. were
used as follows:  S-Lab, a susceptible homzygous refer-
cence strain, 11 and two strains resistant to OP and car-
bamoyl insecticides, homozygous for an insensitive
acetylcholinesterase: MSE, collected in 1979 from
southern France and 12 and Ace-R, collected in 1993 from
Cyprus. 13

C. pipiens possesses two acetylcholinesterases, ACHE1
and ACHE2, which are thought to be produced by dis-
tinct genes, Ace-1 and Ace-2. 11,13 Only Ace-1 is involved
in insecticide resistance and two types of allele can be
distinguished: Ace-1R and Ace-1F, coding for sensitive
and insensitive ACHE1, respectively. 18 The susceptible
strain S-Lab possesses only sensitive ACHE1 enzyme
(and is thus homozygous Ace-1F) and the resistant
strains (MSE and Ace-R) possess only insensitive
ACEH1 (and are homozygous Ace-1R). To obtain het-

erogenous individuals, resistant males of each strain
were crossed with S-Lab females. Offspring were singu-
larly designated as MSE-F1 or Ace-R-F1 depending on the resis-
tant strain used as the male parent.

2.2 Drosophila strains

Three strains of Drosophila melanogaster Meig. were
used, all of which one was a standard susceptible strain
(Canton-S). The other strains (Cha 1 and Cha 2) dis-
played temperature-sensitive CHAT alleles, expressing
low choline acetyltransferase activities. They were
obtained by chemical mutagenesis of the wild-type
strain Canton-S. 19 All strains were reared on standard
medium at 20°C; the restrictive temperature for these
heat-sensitive strains is 25°C.

2.3 Insecticide bioassays

For mosquito strains (and their F1 progeny), resistance
characteristics were analysed by bioassays performed on
fourth instars as described by Raymond and Marquine 20
using the insecticide propoxur (95% pure), which is soluble in water at all concentrations employed
(Bayer, Leverkusen, Germany). In all bioassays, larvae
were exposed to the insecticide for 24 h, and the final
concentration of alcohol was systematically adjusted to
10 ml litre ·1. Each bioassay cup held 20 larvae in
aqueous propoxur (100 mg) and three replicates were
done for each insecticide concentration tested. A
control, where larvae experienced the same environ-
mental conditions except for the presence of the in-
secticide, was run in each experiment. For D. melanogaster
strains, LD50 values were determined for the carbamate
propoxur and the organo-phosphate parathion by tarsal
contact at 20°C. Treatments were performed by putting
10 females in a glass bottle (30 ml capacity, 50 cm2
internal surface) that had previously been soaked with
an acetone solution of insecticide (100 µl). At least five
concentrations of the insecticides were used, giving
between 0 and 100% mortality. Mortality was recorded at
24 h after treatment and LD50 values were determined by
fitting dose/mortality data to sigmoid curves by non-
linear regression using the Prism program.
2.4 ChAT and AChE activity in mosquitoes

AChE activity (i.e. AChE1 plus AChE2 activity) was evaluated using acetylthiocholine iodide as a substrate according to Ellman et al.\textsuperscript{12} Residual activities were recorded after 24 h exposure to propoxur. For the three mosquito strains, S-Lab, MSE and AceR, the three replicates of each dose (i.e. 60 larvae, dead or alive were pooled, washed with water and rapidly mass-homogenized in sodium phosphate buffer (0.1 M, pH 7.0; 1:5 ml) containing Trition X-100 (10 g litre\textsuperscript{-1}), using a glass pestle. Dead larvae did not appear until after 10 h treatment, so that no AChE degradation (by pronase) can occur before activity measurements since this enzyme is very stable over time.\textsuperscript{10} Homogenates were centrifuged (10000 g for 2 min), and mosquito homogenate (100 µl) was added to the substrate-reactant solution (final concentrations: 57-methiocholine-2-nitrobenzoic acid 1.7 mM; acetylthiocholine 3 mM). AChE activity was measured at 412 nm over a period of 1 min using a spectrophotometer (Kontron-Uvikon 930). Assay conditions were established so as to ensure that the rates of enzymatic reaction were linear during the recording period. ChAT activity was recorded as described by Chirico et al.\textsuperscript{14} in the absence and presence during 15 min of three different propoxur concentrations: 20, 200 and 2000 mg litre\textsuperscript{-1}. Activity was measured respectively for 1, 2 and 10 adult mosquito heads of MSE strain homogenized in sodium chloride (0.2 M; 1 ml) containing ' Triton' X-100 (2 g litre\textsuperscript{-1}). Activity was measured after 15 min.

3 RESULTS

3.1 Reduced ChAT activity: an insecticide resistance mechanism

Each of the two Drosophila strains Cha\textsuperscript{61} and Cha\textsuperscript{62} possesses a distinct ChAT structural gene mutant, resulting in a low level of acetylcholine in the central nervous system.\textsuperscript{2} Dose-mortality curves for propoxur and parathion are shown in Fig. 1 along with the curve obtained for the wild-type Canton-S. Temperature-sensitive strains have a two-fold resistance level compared to the reference strain. The resistance ratio was identical for propoxur (a carbamate) and parathion (an organophosphate), which have the same target. Although we cannot exclude the involvement of another gene\textsuperscript{3} present in Cha\textsuperscript{62} mutants in producing the same resistance ratio, this suggests that the resistance observed originates from the decrease of neurotransmitter in the central nervous system which renders the inhibition of AChE by the insecticide less drastic. The two Cha\textsuperscript{62} mutations do not significantly affect neurotransmission at permissive temperature when AChE is not inhibited,\textsuperscript{16} but when neurotransmission is affected by a partial AChE inhibition, a decrease in neurotransmitter synthesis may result in restoring an efficient neurotransmission.

3.2 ChAT: an insecticide target

In the mosquito C. pipiens, several insensitive AChE1 have been described.\textsuperscript{15,16,24,25} Using strains with different AChE sensitivities to propoxur, we studied the remaining enzymatic activity following in-vivo insecticide treatment at concentrations giving from 0 to 100% mortality. For S-Lab (a susceptible strain), and AceR (with a x200-fold less sensitive AChE1)\textsuperscript{12} mortality levels were consistent with AChE inhibition (Figs 2A and 2B). By contrast, for MSE (with x 300/000-fold less sensitive AChE1),\textsuperscript{23} AChE activity was unaffected by the pre-treatment, even at doses of insecticide giving 100% mortality (Fig. 2C). We cannot exclude that AChE activity recovered after extraction. However, the non-inhibition of the AChE enzyme is consistent with the fact that propoxur concentrations giving 100% mortality are far below the concentration needed to inhibit this insensitive AChE1. Thus this result suggests that mortality of MSE larvae is not due to AChE inhibition but to the interaction with another target-site.
Mortality characteristics provide helpful insights on the second target, as the mortality pattern of MSE larvae is different from those of S-Lab and AceR larvae. These two latter undergo violent convulsions, become tetanized and die (ACCh− mortality). To verify this hypothesis, we used nicotine, an ACCh agonist which "mimics" ACCh accumulation. In the presence of lethal doses of nicotine, all mosquitoes (i.e. from S-Lab, AceR and MSE) showed an ACCh− mortality. Conversely, when propoxur was used as insecticide, MSE-resistant mosquitoes did not display convulsion or tetanization; instead they stayed at the surface without moving and progressively shrivelled up and died. We conclude that the inhibition of the second target induced mortality through a physiological process different from ACCh accumulation. These symptoms are tentatively assigned to a defect in neurotransmission arising from a lack of acetylcholine in the synapse (ACCh− mortality), because they resemble the behavioural abnormality of paralysis shown by D. melanogaster strains with reduced ChAT activity.15

Thus, ChAT is a good candidate for the second target, taking in account that some anti-cholinesterase compounds have already been found to inhibit the ChAT enzyme. For example, the anti-cancer drug caracemide, N-acetyl-N-o-diethylcarbamoylhydroxylamine, has been found to inhibit both ACChE by carbamylation of the active site serine16 and ChAT by competition with the substrate acetyl-CoA.17 The ethylcholine mustard, aziridin (AF64A), is also a co-inhibitor of the two enzymes.18 To verify this hypothesis, we tested in vitro the inhibition of ChAT activity by propoxur (Fig. 3). At doses which induced mortality in the MSE strain (Fig. 2C), we observed an inhibition of the ChAT activity by propoxur. Apparently, this inhibition cannot account solely for propoxur mortality in MSE-resistant individuals since in vitro inhibition was not complete, and D. melanogaster mutants were still viable with 8% of wild type activity.19 However, inhibition took place only during 15 min (see Section 2.4) so that the actual in-vivo inhibition may be greater than the observed in-vitro inhibition. More generally this result shows that insecticides analogous to the neurotransmitter acetylcholine may also interact with proteins responsible for the acetylcholine release in the synapse, and hence reduce the neurotransmitter concentration in the synapse. When acetylcholine-receptors are not affected by the insecticide, reduction may become lethal.

Therefore, we tentatively concluded that for S-Lab and AceR mosquitoes, inhibition of their ACChE induces an increase of ACCh at the synapse leading to the ACCh− mortality. Conversely, for MSE insects, the important doses of propoxur probably inhibit ChAT and thus ACCh synthesis. The resulting lack of ACCh release would induce ACCh− mortality.

3.3 ChAT: an insecticide resistance modifier

In MSE-FI mosquitoes (i.e. heterozygous individuals possessing both sensitive and insensitive ACChEs) a complex toxicological phenomenon is observed: within a concentration range of propoxur, mortality decreases when insecticide concentration increases (Fig. 4). This is not an artefact. First, the decrease in mortality is observed systematically within a wide range of insecticide concentrations (10 to 300 mg litre−1), using distinct insecticide stock solutions and in different laboratories.
Second, this puzzling phenomenon has been observed repeatedly since 1985 and was found for three resistant strains possessing the same insensitive allele but different genetic backgrounds (data not shown). Third, a decrease in mortality when propoxur doses increase has also been observed in field samples, where heterozygous mosquitoes are at relatively high frequencies.10

This phenomenon is unusual and has been described, as far as we know, only twice in the literature: in Macrophomina phaseolina Cav. resistant to the fungicide tetramethylthiuram disulfide (Dmond et al., 1941, in Finney39) and in Venteria impressa (Cooke), Wnt. resistant to thiuram sulfoxide.36 For the first case, the decrease in resistance with increasing doses was explained by the dose-dependent dissociation of the fungicide into molecules of higher toxicity.29,30 This explanation does not apply for the present observations, as dissociation of propoxur into more toxic compounds is unlikely (Fuxuto, pers. comm.).

This unusual phenomenon could be explained by the inhibition of the ChAT as previously described for MSE, MSE-F1 hybrids possessing both sensitive and insensitive AChE1 enzymes. When propoxur doses increase, there is a progressive inhibition of the sensitive fraction until only the insensitive AChE1 remains. This insensitive fraction alone is not sufficient to decrease the ACh accumulation and ACh* mortality is observed. Indeed, resistant AChE1 is altered and has only 21% of wild type activity at 1 nm of substrate.45 Thus, in heterozygotes, when the susceptible counterpart is inhibited, the remaining AChE1 activity is near the minimum for viability. At higher doses, propoxur starts to inhibit the ChAT (see above). The resulting decrease in ACh synthesis could balance the previous ACh accumulation and explain the observed decrease in mortality. When propoxur doses continue to increase, ACh* mortality appears to be due probably to the complete inhibition of the ChAT.

Conversely, AceR-F1 heterozygote mortality curves do not show any decrease when propoxur concentrations increase. This is because both sensitive and insensitive AChE1 are inhibited at lower propoxur concentrations than the ChAT so that mortality is always the result of ACh accumulation. As expected, for AceR-F1, only ACh* mortality is observed.

4 DISCUSSION

We have taken advantage of mutant ChAT flies and mosquitoes with a fully insensitive AChE1 to estimate the influence of ChAT and the interaction between AChE and ChAT on insecticide resistance. Our results suggest that a reduction of choline acetyltransferase is a potential resistance mechanism (as shown in mutant D. melanogaster), and that ChAT represents also a pesticide target-site (as for example in mosquitoes with insensitive AChE1) or a modifier of insecticide resistance (in heterozygotes, where it probably induces a mortality decrease when insecticide concentration increases).

Resistance to insecticides results from three main physiological mechanisms: reduced penetration, increased detoxification or reduced target sensitivity.54 Here, we have found another potential mechanism which has not been described as yet, namely the underproduction of the target’s substrate (here through a
reduced ChAT activity). This modification is a resistance mechanism when excess of the target's substrate is lethal, as it is the case for the AChE target. This situation confirms that the only relevant effect of organophosphate and carbamate toxicology is on the neurotransmitter level, which can be decreased either by over-production of acetylcholinesterase, or by under-production of ChAT (this study), the two changes resulting in insecticide resistance. Apparently, this mechanism confers only a low resistance level in D. melanogaster, but in the presence of other resistance mechanisms, it may contribute additively or multiplicatively to a higher resistance level. Since there is no direct binding between the insecticide and the ChAT, the resistance mechanism of reduced ChAT activity could potentially exist for all carbamates and organophosphates (i.e. to approximately half of insecticides used currently in the world).

Toxicological studies state that insecticide toxicity is due to the action on only one major target-site, either the GABA receptor, acetylcholinesterase or sodium channel for most common insecticides. Thus OPs and carbamates act on acetylcholinesterase and owe their toxicity to their effects on this enzyme. This was indicated by the correlation between thoracic AChE inhibition and poisoning, and the link between AChE modification and insecticide resistance. However, in vivo, insecticides may affect several other interacting target-sites. Since these targets are less sensitive to insecticides, they are normally not relevant to explain insect mortality. But when the main target becomes less sensitive, higher insecticide concentrations are required and secondary targets may be involved. Here, we show that, in vivo, ChAT may be an alternative insecticide target-site for at least a carbamate insecticide (propanil).

Organophosphorus and carbamate insecticides are substrate analogues and modications giving less sensitive AChE usually result in a partial decrease in substrate metabolism. This alteration in acetylcholine metabolism may be quite important. For example, the MSe strain used in this study presents only 21% of the AChE1 wild type activity in the apparent Vm. This remaining AChE1 activity is sufficient for life under laboratory conditions and may even be decreased to much lower levels, as shown by the residual activity following insecticide treatments or rescue experiments with an injected minigene in D. melanogaster. However, in natural conditions, a fitness cost seems to be associated with the mosquito's insensitive allele. It appears that ChAT under-production is a good candidate to decrease the deleterious effect on fitness produced by the AChE1 alteration. Such a balancing effect by mutations in an enzyme that restore the steady state level disturbed by a mutation in another enzyme was already described. For example, in Escherichia coli Castell & Chalm., top A (the gene coding the topoiso-
merase I) mutants are viable only if they acquire compensating mutations that reduce the level of grn. In the case of AChE and ChAT enzymes, each alteration compensates the physiological effect of the other. ChAT mutations may thus also be considered as a potential modifier that allows maintenance of altered mutations in untreated areas. Under-production of an enzyme such as ChAT must be quite frequent; many mutations may result in a decreased activity, ria, for example, under-transcription or inefficient folding. But we may expect insecticide treatments to select another kind of mutation: a modified ChAT with a higher sensitivity to insecticides. In insects harbouring a wild-type AChE, pest control would favour a ChAT more sensitive to insecticides because the co-inhibition of the two enzymes would confer insecticide resistance. On the other hand, when insects display an insensitive AChE, insecticide treatments would select for a less sensitive ChAT enzyme. These ChAT modifications may arise by point mutations modifying the active site, such as those previously described for AChE2. In ChAT only a few amino-acid changes render the AChE less sensitive while retaining enough enzyme activity for an efficient neurotransmitter metabolism. The same constraint would apply for ChAT modifications: whatever the mutations, the enzyme activity should remain in sufficient amount to maintain the AChE above the lower threshold. We therefore predict that studying in detail the ChAT enzyme or gene will lead to better understanding of the evolution of insecticide resistance in natural populations.

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