LEVELS OF ORGANOPHOSPHORUS AND CARBAMATE INSECTICIDE RESISTANCE CONFERRED BY INSENSITIVE ACETYLCHOLINESTERASE IN THE BEET ARMYWORM, *SPODOPTERA EXIGUA* (HUBNER)

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Abstract

Two forms of acetylcholinesterase (AChE) were identified in field populations of the beet armyworm, *Spodoptera exigua* (Hubner) collected from cotton in San Joaquin Valley, California. Strains (BESS and BKRR) homogeneous for each variant were isolated and their relative susceptibilities to methomyl, chlorpyrifos and chlorpyrifos-oxon assessed by topical application bioassay. In comparisons with a laboratory susceptible strain (DOW), BKRR and BESS expressed 68-fold and 7-fold resistance, respectively, to the carbamate methomyl. Neither strain was cross-resistant to chlorpyrifos or its oxygen analog (chlorpyrifos-oxon). In biochemical studies, the BKRR AChE enzyme was *ca*. 30-fold and 7-fold more insensitive to methomyl and chlorpyrifos-oxon, respectively, compared with both the DOW and BESS enzymes. The correlation between the toxicological and biochemical studies provides strong evidence that target-site insensitivity is the predominant mechanism of resistance to methomyl. The lack of significant cross-resistance to chlorpyrifos suggests also that the insensitive AChE in these field populations was selected by methomyl alone and not by the OP.

Introduction

Methomyl (carbamate) and chlorpyrifos (organophosphate) are routinely used for the control of the beet armyworm (BAW), *Spodoptera exigua* (Hubner), on cotton in California. Although widespread tolerance to these compounds has been well documented in Californian populations of BAW (Brewer and Trumble, 1994; Kerns *et al.*, 1998; Mascarenhas *et al.*, 1998; Sparks *et al.*, 1996), little information is available on the biochemical mechanisms involved in conferring reduced susceptibility (Byrne *et al.*, 1999).

The aims of this study were to use toxicological and biochemical methods to determine the levels of OP and carbamate resistance associated with different AChE enzymes expressed in *S. exigua*. Chlorpyrifos and chlorpyrifos-oxon were used in bioassays to determine whether the rates of activation of the OP to the active oxygen analog were similar in all strains or could form a potential source of resistance.

Materials and Methods

Insects

The Dow AgroSciences laboratory-reared strain of BAW was used as the reference susceptible strain in all bioassay and biochemical experiments. Field populations were collected in Sept., 1998, from cotton fields on Birkshire Road, Bakersfield, California. All insects were reared on artificial diet (Patana, 1985) at 80 °F under a 16:8 (L:D) photoperiod.

Insecticides

Technical grade methomyl (98%; DuPont Agricultural Products), chlorpyrifos (99.8%; Dow AgroSciences) and chlorpyrifos-oxon (96%; Dow AgroSciences) were gifts from their respective manufacturers, and were used for both toxicological and biochemical components of the study.

Toxicological Bioassays

Test solutions of technical grade insecticides were prepared in 100% acetone immediately prior to bioassays. Insecticide (in 0.5µl) was topically applied to the dorsum of the thorax of 3rd instar larvae using an Arnold hand applicator (Burkard, Rickmansworth, Herts, U.K.). 25 insects (8-12mg) were treated at each test concentration and mortality was assessed after 48h. Control insects were included in each bioassay and were treated with acetone only. Probit analyses of concentration-dependent mortality were done using POLO-PC (LeOra Software, 1987).

Measurement of AChE Activity

All *in vitro* AChE measurements were done using adult heads as the enzyme source. Activity was measured by the method of Ellman *et al.* (1961), in which the hydrolysis of the natural substrate analog ATChI was determined colorimetrically by the absorbance of 2-nitro-5-thiobenzoate (TNB) at 405 nm after the reaction of DTNB with the liberated thiocholine. Prior to all assays, the supernatants were incubated with 100µM DTNB for at least 15 min at 4 °C to neutralize the effects of free thiols on the detection of AChE activity (Byrne and Devonshire, 1997). All measurements were made at 25 °C in a SpectraMAX 250 kinetic microplate reader (Molecular Devices Corp.).

Determination of K_M

Mass homogenates of 4 adult heads were prepared in 0.1M phosphate buffer, pH 7.5, containing 0.1 % Triton X-100. These were centrifuged at 5,000g and 4 °C for 15 min. in a Hermle Z 360 K refrigerated centrifuge. Values of K_M and V_{max} were determined at 25 °C from AChE activities measured over 1 min for 16 ATChI concentrations ranging from 0.025 to 50 mM using the equivalent of 0.05 of a single head per assay.

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Inhibition Kinetics

For all inhibition experiments, homogenates of 5 adult heads per ml were prepared in phosphate buffer containing Triton X-100. Inhibition of AChE activities by methomyl and chlorpyrifos-oxon was determined in the presence and absence of substrate. For measurements in the presence of substrate (co-incubation method), a series of solutions of ATChI/DTNB (0.75mM/0.15mM) in buffer (200µl) containing increasing concentrations of insecticide were added to enzyme (100µl; equivalent to 0.025 of a single head) in the wells of a microtiter plate. Activity was monitored continuously for 30 min at 25 °C in the SpectraMax 250 microplate reader. The percentage activity remaining in each well was determined from the slopes of the kinetic plots and these were then used to derive the insecticide concentration required to inhibit 50% of activity (I_{50}). Methomyl and chlorpyrifos-oxon were tested over the concentration ranges 0.1 – 1000 µM and 0.003 – 30 µM, respectively.

For measurements of inhibition in the absence of substrate (Aldridge and Reiner, 1972), eight insecticide concentrations (in 10 µl buffer) were added to the wells of a single column of a microplate and equilibrated at 25 °C in the reading chamber of the SpectraMax 250. To begin inhibition reactions, 190 µl aliquots of enzyme (equivalent to 0.855 of a single head) were added simultaneously to the wells containing insecticide. After various periods of incubation, aliquots (20 µl) were removed and transferred to the adjacent wells of the same microplate. Immediately upon transfer, further inhibition was terminated by the addition of 250 µl of ATChI/DTNB (final concentrations of 1mM/100µM) and residual AChE activity measured for 1 min.

Kinetic Analyses

Values of K_M and V_{max} were determined by computing a least-squares fit of the data to the Michaelis-Menten equation $v = V_{max}.s/K_M+s$ using EnzFitter software (Leatherbarrow, 1987; Wilkinson,1961). Bimolecular rate constants (k_i) were determined directly from plots of pseudo-first-order rate constant (k) against inhibitor concentration using the same least-squares approach.

Results

Isolation of Homogeneous Strains

Two AChE variants, differing in their responses to inhibition by 30 µM methomyl, were identified in BAW populations collected from cotton crops in Bakersfield, California in 1998 (Byrne *et al.*, 1999). The progeny arising from single-pair matings between parents that were homozygous for the same genotype were pooled to form homogeneous strains for the sensitive (BESS) and insensitive (BKRR) variants.

Expression of Resistance in Bioassays

The upper practical dose for topical application of methomyl in 0.5 µl acetone was 100 µg per larva. As a result of this dose limit, it was not possible to derive a complete dose-response curve for BKRR, and estimates of the upper 95% confidence limit for BKRR are, therefore, extremely high (Table 1).

In comparisons with the DOW susceptible strain, LC_{50}s for BESS and BKRR with methomyl were 7-fold and 68-fold higher, respectively (Table 1). In contrast, chlorpyrifos and its O analog were extremely toxic to all strains, including BKRR, which expressed only a 2-fold resistance factor to both chemicals relative to DOW and BESS. However, even this low level of resistance in BKRR proved to be significantly different from DOW and BESS, reflecting the narrow dose range required to obtain full dose-response lines for all strains (Tables 2-3).

Characterization of AChE Enzymes

Figure 1 shows the inhibitory effects of methomyl and chlorpyrifos-oxon on enzyme activity from DOW, BESS and BKRR measured in the presence of substrate. Although DOW and BESS were at least 200-fold more sensitive to the OP than the carbamate, these strains could not be distinguished using any individual compound (Table 4). In contrast, BKRR was more insensitive to both insecticides, particularly methomyl, and could be readily distinguished from DOW and BESS in microplate assays of individual adult heads at a 30 µM diagnostic discriminating concentration of methomyl (Fig. 1).

Measurements of inhibition in the absence of substrate (Table 5) also showed that chlorpyrifos-oxon was the most potent inhibitor of enzyme activity in all three strains, reflecting its greater toxicity in bioassays (Table 3). The BKRR AChE again proved to be the most insensitive enzyme with an insensitivity ratio (IR) to methomyl of 31-fold relative to DOW. The inhibition of the BESS enzyme was similar to that from DOW with both the OP and carbamate, again providing a good correlation between the bioassay and biochemical data.

Measurements of K_M (Table 6) showed that the DOW AChE had the highest affinity for the substrate analog ATChI (K_M = 74µM). The BESS enzyme, which could not be reliably distinguished from the DOW enzyme based on inhibitory characteristics, had an approx. 8-fold lower affinity as judged by its higher K_M of 568µM, providing conclusive evidence that the two enzymes are different. The BESS enzyme could also be distinguished from DOW by virtue of its greater activity (expressed in terms of V_{max} in Table 6). The kinetic data for BKRR showed it to be the most active enzyme of the three tested and to have intermediate affinity for substrate (Table 6).

Discussion

In BAW populations collected in 1998 from the San Joaquin Valley, California, two AChE variants were identified which differed in their sensitivities to inhibition by methomyl. Following the development of strains homozygous for the sensitive (BESS) and insensitive (BKRR) variants, the expression of resistance in topical bioassays was consistent with the presence of the insensitive AChE, providing the first direct evidence for target-site resistance to methomyl in BAW. Methomyl has been used extensively to control BAW in California and other states (Aldosari *et al.*, 1996; Brewer and Trumble, 1994) in the U.S., and resistance to this insecticide is now widespread.

Resistance in BKRR was much higher for methomyl (68-fold) than for either chlorpyrifos (2-fold) or its O analog (2-fold) indicating the dominant role of the carbamate in selecting the insensitive AChE. This was corroborated by the biochemical data, which highlighted significant insensitivity to methomyl in the BKRR enzyme. There was little difference between the toxicological responses of BKRR and the two susceptible populations (DOW and BESS) towards chlorpyrifos, indicating a lack of significant cross-resistance to the OP. However, there was 7-fold insensitivity to the OP when inhibition was measured in the absence of substrate and this probably accounts for the mild (2-fold) tolerance observed in bioassays.

It is interesting to note that, for each strain, there was little difference in the concentrations of insecticide required to inhibit AChE activity in the presence or absence of substrate. This indicates that the combined effects of substrate affinity (K_M) and enzyme activity (V_{max}) on the interaction between the BAW enzymes and the OP or carbamate, do not significantly enhance or diminish the influence of the intrinsic insensitivity, as occurs in other pest species (Byrne and Devonshire, 1997; Devonshire and Moores, 1984). This is most evident in comparisons of kinetic data for DOW and BESS, which have enzymes of equal sensitivity to both methomyl and chlorpyrifos-oxon. BESS has a *ca.* 8-fold less efficient enzyme in terms of its affinity for substrate, which would be expected to reduce the relative protective effect of substrate on the enzyme. However, its V_{max} is *ca.* 3-fold higher and this seems to be sufficient to mitigate any deleterious effects arising from poor affinity. In bioassays, BESS expressed a 7-fold greater tolerance to methomyl than DOW, suggesting that in cases where levels of insensitivity are the same, the critical feature is not the affinity for substrate, but the overall activity of the enzyme. A higher enzyme activity means that a greater proportion of enzyme must be inhibited before the critical threshold for survival is reached.

Chlorpyrifos requires activation to its oxygen analog in order to achieve its toxic effect. Against each strain, the O-analog was approx. 2-fold more toxic than the parent compound indicating similar *in vivo* rates of activation. BKRR expressed a 2-fold tolerance to both chemicals compared to DOW, and while this is unlikely to constitute a major resistance risk, the data show that the rates of activation of the OP do not contribute to that resistance.

We have shown in this study, that levels of resistance to methomyl attributable to insensitive AChE can be as high as 68-fold, when toxicity is measured relative to the DOW laboratory susceptible. In field populations expressing the BKRR AChE, levels of resistance above 68-fold will indicate the presence of additional mechanisms, provided that the same susceptible standard is used in all measurements. When this is not possible, implicating a role for other mechanisms can be more reliably done using comparisons of LC_{50} data. We also found that resistance to chlorpyrifos was not significant in our resistant field strain; in fact, chlorpyrifos was over 700-fold more toxic to BKRR than methomyl, and it seems likely, therefore, that reports of poor control with this insecticide are due to either the presence of additional mechanisms or poor application. Additional mechanisms can be inferred by comparing the current data with those from an earlier study on populations from the same cotton fields. Byrne et al. (1999) reported an LC_{50} (0.55 μg/larva) for chlorpyrifos-oxon in one field strain which was 3-fold higher than that reported in this study for BKRR. The BESS and BKRR AChE variants were both present in that field population at approximately equal frequencies, indicating that a mechanism other than AChE insensitivity was involved in conferring the higher resistance.

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Table 1. Responses of three *S. exigua* strains to methomyl in topical bioassays.

Strain	LC_{50} (μg/L_3)	95% Conf. Limits	Slope	S.E.	RF
DOW	3	1.7-5.4	0.97	0.15	1
BESS	20	18-23	5.4	0.98	7
BKRR	205	109-2721*	1.4	0.42	68

Table 2. Responses of three *S. exigua* strains to chlorpyrifos in topical bioassays.

Strain	LC_{50} (μg/L_3)	95% Conf. Limits	Slope	S.E.	RF
DOW	0.178	0.15-0.209	7.26	1.198	1
BESS	0.170	0.135-0.211	3.291	0.599	1
BKRR	0.287	0.244-0.341	4.848	0.796	2

Table 3. Responses of three *S. exigua* strains to chlorpyrifos-oxon in topical bioassays.

Strain	LC_{50} (μg a.i./L_3)	95% Conf. Limits	Slope	S.E.	RF
DOW	0.097	0.074-0.13	2.286	0.32	1
BESS	0.078	0.064-0.096	3.029	0.478	1
BKRR	0.169	0.133-0.221	2.448	0.319	2

Table 4. I_{50}s (μM) for inhibition of AChEs in adult *S. exigua*. Values are the means of at least two independent determinations. SEM in parentheses.

Strain	Methomyl	IR*	Chlorpyrifos-oxon	IR
DOW	6 (0.5)	1	0.03 (0.01)	1
BESS	9 (1.0)	1.5	0.04 (0.01)	1.3
BKRR	113 (13)	19	0.07 (0)	2.3

* IR, Insensitivity Ratio calculated by dividing the I_{50} for each strain by the I_{50} for the DOW strain.

Table 5. Bimolecular rate constants (k_i, $mM^{-1}min^{-1}$)[a] for inhibition of AChE in adult *S. exigua*. Values are the means of at least two independent determinations. SEM in parentheses.

Strain	Methomyl	IR*	Chlorpyrifos-oxon	IR
DOW	34 (5)	1	1834 (74)	1
BESS	48 (4)	0.7	2874 (61)	0.6
BKRR	1.1 (0.1)	31	268 (20)	7

*IR, Insensitivity Ratio calculated by dividing the k_i for the DOW strain by the k_i for the field strains.

Table 6. K_M (μM) and V_{max} (mOD/min/head) of AChE in adult *S. exigua* for the natural substrate analog ATChI. Values are the means of at least two independent determinations. SEM in parentheses.

Strain	K_M	V_{max}
DOW	74 (9)	592 (36)
BESS	568 (63)	1493 (130)
BKRR	164 (21)	1660 (183)

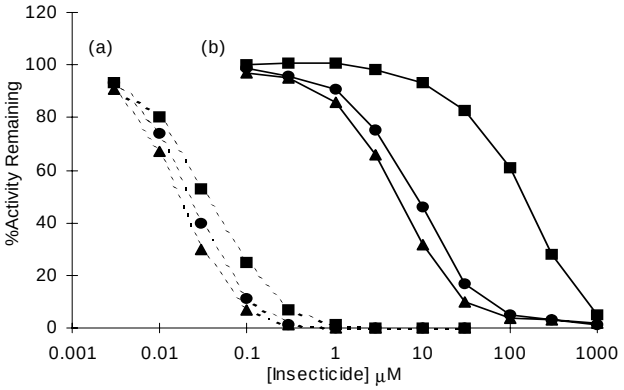

Figure 1. Inhibition of BAW acetylcholinesterases by (a) chlorpyrifos-oxon and (◆) methomyl in DOW (●), BESS (■) and BKRR (n) *S. exigua*.