有機リン剤抵抗性イエバエの変異型アセチルコリンエステラーゼに対するサリオキソンとフェニトロオキソンの効果

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Original Article

Effects of Salioxon and Fenitroxon on Altered Acetylcholinesterase of Organophosphate-Resistant Housefly

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Interaction between organophosphates (OP) and housefly acetylcholinesterases (AChE) from the susceptible SRS strain and OP-resistant Daisan Yumenoshima (3-Y) strain was investigated. The ratios (3-Y/SRS) in half inhibitory concentration (I_{50}) of fenitroxon and salioxon were 46.2 and 4.6, respectively. This difference is the main reason of salithion's effectiveness against OP-resistant houseflies; the R/S ratios in the half lethal doses (LD_{50}) of fenitrothion and salithion were 259 and 8.2, respectively. The affinity of fenitroxon ($1/K_d$= 0.77 mM^{-1}) for 3-Y AChE was much lower than that of salioxon ($1/K_d$=3.55 mM^{-1}). Fenitrothion took longer to kill OP-resistant flies and to inhibit their AChE *in vivo* than salithion. Fenitroxon-inhibited AChE's of both strains were reactivated more than 10% *in vitro* with an oxime-type reactivator, whereas salioxon-inhibited AChE's less than 5%.

INTRODUCTION

Among the organophosphate(OP)-resistant factors, the insensitivity of acetylcholinesterase (AChE) and the high activity of glutathione *S*-transferase are most important.[1] In order to clarify the effectiveness of some organophosphorus insecticides against OP-resistant houseflies, interaction between OP and AChE was investigated. Since it has been reported that salithion is much more effective than fenitrothion against OP-resistant houseflies,[2] the potency of fenitroxon and salioxon was compared.

MATERIALS AND METHODS

1. *Chemicals*

Fenitrothion and salithion were supplied by Sumitomo Chemical Co., Ltd. Fenitroxon was prepared as follows: To a stirred solution of 1.0 g (3.6 mmol) of fenitrothion in 20 ml of dichloromethane, 1.0 g (60% excess) of 85% *m*-chloroperbenzoic acid was added at 0°C. After 3 hr, 5 ml of 10% sodium sulfate was added to the reaction mixture and *m*-chlorobenzoic acid was removed by washing with 10 ml of 5% sodium bicarbonate. The separated dichloromethane layer was evaporated under reduced pressure. The residue obtained was submitted to a silica gel column chromatography, through which fenitroxon was purified using a benzene–ethyl acetate mixture (1 : 1) as an eluent. Salioxon was synthesized by condensation of saligenin with methyl phosphorodichloridate in chloroform containing pyridine as a catalyst.[3] Monoisonitrosoacetone (MINA) was prepared according to Freon's method.[4]

2. *Test Insects and Insecticidal Test*

The SRS strain represented a susceptible strain and the Daisan Yumenoshima (3-Y) strain as an OP-resistant strain. Both strains were kindly supplied by Dr. T. Shono of the University of Occupational and Environmental Health. Insecticidal tests were carried out by topically applying the acetone solution of test chemicals (1 μl) to the dorsal thorax of

ten female flies (3 to 4-day old). The flies were kept at 25°C and their mortality was counted 24 hr after treatment.

3. Enzyme Preparation

Fly heads were homogenized with a teflon-glass Potter-Elvehjem tube in ice-cold 0.1 M phosphate buffer, pH 8.0 (10 heads per ml unless otherwise noted). The homogenates were centrifuged at $2500\times g$ for 10 min at 0°C, and the supernatant was again centrifuged at $10,000\times g$ for 10 min at 0°C. The supernatant thus obtained was used as a fly AChE preparation.

4. Inhibition Test for AChE

Ellman's spectrometric method[5] was applied for assays of AChE activity with slight modifications. A mixture containing 2.85 ml of 0.1 M phosphate buffer, pH 8.0, 0.1 ml of Ellman's reagent [0.01 M 5,5'-dithiobis(2-nitrobenzoic acid) and 17.8 mM sodium bicarbonate in 0.1 M phosphate buffer, pH 7.0], and 0.15 ml of the fly AChE was preincubated at 25°C for 2 min. Thirty-two microliters of inhibitors in acetone was added to the mixture, and the inhibitor-added mixture was incubated at 25°C. After 15 min, 32 μl of 75 mM acetylthiocholine iodide (ATCh) was added to the mixture and the change in absorbance at 412 nm was measured by a Shimadzu UV-200 spectrophotometer. Concentration required to inhibit the enzyme reaction by 50% (I_{50}) was calculated from the velocity of ATCh hydrolysis. Control experiments were performed with an enzyme solution containing the same volume of acetone as above without inhibitors.

5. Enzyme Kinetics

5.1 The Michaelis constant and maximum velocity

The Michaelis constant (Km) and maximum velocity (V_{max}) were calculated from rates of hydrolysis of ATCh or butyrylthiocholine iodide (BuTCh) at six or more concentrations. The velocity of ATCh hydrolysis was measured as described above without adding inhibitors. BuTCh hydrolysis was measured similarly by adding BuTCh instead of ATCh. The kinetic constants were calculated by using non-linear regression analysis.[6]

5.2 The dissociation constant of the enzyme-inhibitor complex and phosphorylation constant

The dissociation constant (K_d) of the enzyme-inhibitor complex and phosphorylation constant (k_2) were determined according to the method described by Hart and O'Brien,[7] using non-linear regression analysis. And the bimolecular reaction constant (k_i) was calculated from k_2/K_d as postulated by Main.[8]

6. In Vivo Inhibition of AChE

Three hundred and twenty houseflies each treated topically with 1 μl of OP almost equivalent to LD_{50} in acetone were kept at 25°C (20 flies in a petri dish). At optional intervals, their mortality was counted for two batches (40 flies) and the heads of survived flies were used to prepare AChE solutions. AChE activity was measured as described above with no further addition of inhibitor.

7. In Vitro Reactivation Test

After the fly AChE solution (2 ml, 20 heads/ml) was incubated with an inhibitor at 5×10^{-6} M at 25°C for 1 hr, the excess inhibitor was removed from the reaction mixture by gel filtration (Sephadex G-25, 2×25 cm) using 0.1 M phosphate buffer, pH 8.0, as an eluent. Protein fractions monitored by absorbance at 280 nm were combined (*ca.* 20 ml) to be a sample of OP-inhibited AChE. A 2-ml aliquot was incubated with a 1 ml an aqueous 0.1 M MINA solution at 25°C for 1 hr. Excess MINA was removed by gel filtration as mentioned for removing the inhibitors, and a 3-ml aliquot of each fraction (4 ml) was used as MINA-induced reactivated AChE. The OP-inhibited AChE (2 ml) was incubated at 25°C for 12 hr to obtain spontaneously reactivated enzyme. This enzyme solution was gel-filtered before AChE assay as in the case of the MINA-induced reactivation test. The control sample of AChE was also gel-filtered twice as in the test. The ratios of AChE activity to absorbance at 280 nm was calculated.

RESULTS AND DISCUSSION

1. Insecticidal Activity

The insecticidal activity of fenitrothion and salithion against SRS and 3-Y houseflies is

shown in Table 1. The 3-Y strain was highly resistant to fenitrothion and comparatively susceptible to salithion.

2. Enzyme Kinetics

Kinetic constants for the hydrolysis of ATCh and BuTCh are presented in Table 2. Against both substrates, a slightly lower affinity was observed for the 3-Y strain than for the SRS strain. The V_{max} value of the 3-Y against ATCh was, however, remarkably high in comparison with that in other combinations. Table 3 shows I_{50} values of fenitroxon and salioxon against AChE of SRS and 3-Y houseflies. The AChE of the 3-Y strain was 46-fold less sensitive toward fenitroxon than that of the SRS strain, but only 4.6-fold toward salioxon. It is, therefore, presumed that this difference in sensitivity is the main reason of salithion's effectiveness against resistant houseflies. The affinity ($1/K_d$), and phosphorylation (k_2) and bimolecular reaction (k_i) constants for the inhibitory reaction of fly-head AChE with fenitroxon and salioxon are summarized in Table 4. Judging from $1/K_d$, the affinity of fenitroxon for 3-Y AChE stays very low (R/S value=0.023), while the affinity of salioxon decreases a little (R/S=0.15). Difference in k_2 is not so big as in $1/K_d$ within any combinations of the present strains and inhibitors. Thus, k_i values show mainly the difference in affinity. It is, therefore, concluded that relatively high inhibitory activity of salioxon against the AChE of the 3-Y strain is due to a smaller decrease in affinity of salioxon for the OP-resistant fly AChE.

3. In Vivo Inhibition of AChE

The time courses of mortality and residual AChE activity of the insects treated with fenitrothion or salithion equivalent to LD_{50} are shown in Fig. 1. For SRS flies, there was no significant difference between fenitrothion and salithion in time required for 50% AChE inhibition (IT_{50}=70–80 min). Thirty percent of SRS flies were killed within 160 min by both insecticides. For the salithion-treated 3-Y strain, IT_{50} and the 30% lethal time were 160 min. For the fenitrothion-treated 3-Y strain, however, IT_{50} was about 220 min after treatment and it took 640 min to reach the 30% lethal level. There was a considerable delay until the fenitrothion-treated 3-Y flies showed fatal effects. This delay as well as the difference

Table 1 Insecticidal activity of fenitrothion and salithion against SRS and 3-Y houseflies.

	LD_{50} (μg/fly)		R/S
	SRS	3-Y	
Fenitrothion	0.07	18.16	259
Salithion	0.06	0.49	8.2

Table 2 Kinetic constants for ATCh and BuTCh hydrolysis.

Kinetic constant	SRS	3-Y	R/S
ATCh			
Km (μM)	134	284	2.12
V_{max} (μM·min^{-1}·mg protein^{-1})	43.3	500.7	11.54
BuTCh			
Km (μM)	143	227	1.59
V_{max} (μM·min^{-1}·mg protein^{-1})	43.3	90.6	2.09

Table 3 Inhibitory activity of fenitroxon and salioxon on ATCh hydrolysis by housefly head AChE.

Inhibitor	I_{50} (nM)		R/S
	SRS	3-Y	
Fenitroxon	93	4300	46.2
Salioxon	140	650	4.6

Table 4 Kinetic constants for ATCh hydrolysis inhibition by fenitroxon and salioxon.

Kinetic constant	SRS	3-Y	R/S
$1/K_d$ (mM^{-1})			
Fenitroxon	33.3	0.77	0.023
Salioxon	23.8	3.55	0.149
k_2 (min^{-1})			
Fenitroxon	6.24	10.37	1.66
Salioxon	8.69	13.09	1.51
k_i (mM^{-1}·min^{-1})			
Fenitroxon	207.8	7.98	0.038
Salioxon	206.8	46.5	0.225

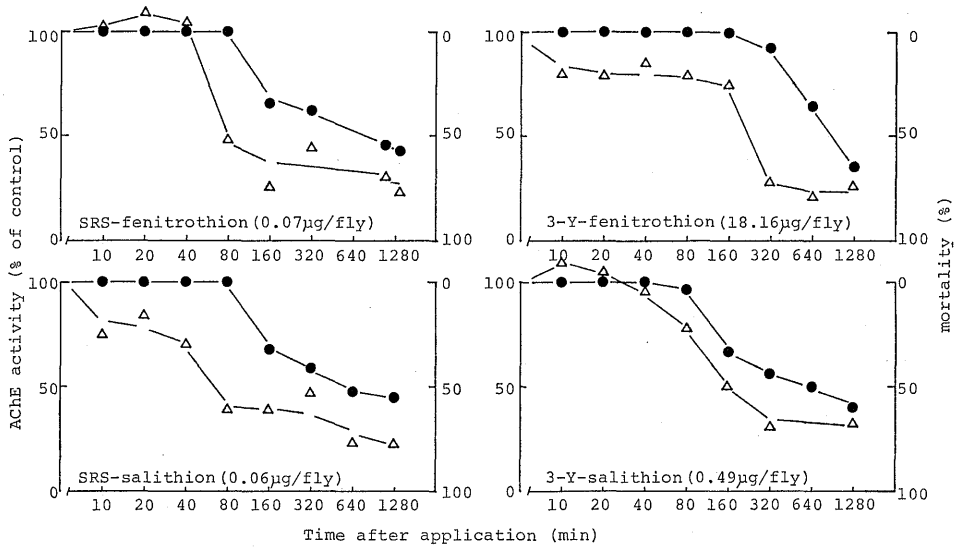

Fig. 1 Time courses of mortality (●) and residual AChE activity (△) of susceptible (SRS) and OP-resistant (3-Y) houseflies.

Table 5 Spontaneous or MINA-induced reactivation of OP-inhibited housefly head AChE.

	ATCh hydrolysis ($nM \cdot min^{-1} \cdot A_{280}^{-1}$)	
	SRS	3-Y
Control	796	909
Fenitroxon-inhibited		
0 hr	2	4
+MINA (1 hr)	101	104
−MINA (12 hr)	13	53
Salioxon-inhibited		
0 hr	1	1
+MINA (1 hr)	7	42
−MINA (12 hr)	—	2

in R/S ratios of LD_{50} (259, see Table 1) and I_{50} (46, see Table 3) suggests that factors other than the insensitivity of AChE, such as metabolic ability on fenitrothion, may contribute to the OP-resistance mechanism of 3-Y strain houseflies. To clarify this point further study is now in progress.

4. Reactivation of Inhibited AChE

Since it has been known that some oximes including MINA reactivate phosphorylated serine enzymes,[9] reactivation of OP-treated fly-head AChE with MINA was investigated. The fenitroxon-treated AChE was reactivated more than 10% by MINA in both SRS and 3-Y strains, whereas spontaneous reactivation is about 2% and 6% in the SRS and 3-Y strain, respectively (Table 5). On the other hand, salioxon-treated AChE of the 3-Y strain was reactivated about 5% by MINA but not spontaneously (<1%). Salioxon-treated AChE of the SRS strain was reactivated less than 1% even by further MINA treatment. This may also be one of the possible reasons for insecticidal potency of salithion against the resistant flies.

This study is summarized: AChE of the OP-resistant 3-Y strain was highly insensitive owing to its low affinity. On the other hand, salithion was effective against OP-resistant houseflies. The possible reasons are: (1) Salioxon has a comparatively high inhibitory activity against the AChE of OP-resistant houseflies, (2) the affinity of salioxon for the AChE is higher than that of fenitroxon, (3) 50% inhibition of AChE of OP-resistant houseflies *in vivo* is attained faster by salithion than by fenitrothion, and (4) salioxon-inhibited AChE is hard to be reactivated.

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要　　約

有機リン剤抵抗性イエバエの変異型アセチルコリンエステラーゼに対するサリオキソンとフェニトロオキソンの効果

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第三夢の島系統イエバエの有機リン剤抵抗性は，アセチルコリンエステラーゼ(AChE)のリン剤に対する感受性が低下しているのがその一因であった．サリチオンがこの系統のイエバエに殺虫剤として比較的有効であるのは，主として，酸化体サリオキソンに対する感受性の低下が，フェニトロオキソンに比べ小さいことによる．これは，各リン剤の AChE に対する親和性 ($1/K_d$) の低下に差違が生じたことによることがわかった．抵抗性イエバエにフェニトロチオンを局所施用した時の致死効果および AChE 阻害効果の発現は，感受性の場合に比べ遅延したが，サリチオンでは，感受性・抵抗性間に差がなかった．また，サリオキソンにより阻害された AChE は，フェニトロオキソンにより阻害された AChE と比較し，自然にあるいはある種のオキシムの作用によって回復しにくかった．