PESTICIDE ACTION AND MEMBRANE FLUIDITY

Allosteric behavior of rat erythrocyte membrane-bound acetylcholinesterase in the presence of organophosphorous compounds

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1. Introduction

It is well documented that the organophosphorous pesticides or their metabolites are powerful inhibitors of acetylcholinesterase with consequent disruption of nervous activity [1–2].

Variations in the interaction energies as low as 700 cal/mol would be enough to trigger significant changes in the Hill coefficient from membrane-bound enzymes (n-values) [3]. It was indicated that the cooperative behavior of several membrane-bound enzymes correlated with the membrane fluidity and that these relationships raised the possibility of evaluating changes in membrane fluidity [4]. This novel approach was illustrated in the case of cholesterol [5], cortisol, progesterone [6], insulin [6–7] and thyroid hormones and thyrotropin interplay [8].

In this work we offer biochemical evidence regarding a new molecular basis in the action of organophosphate agents on the membrane fluidity through studies of the allosteric behavior of the inhibition by F^- of erythrocyte membrane-bound acetylcholinesterase (EC 3.1.1.7) from rats fed a corn oil or lard supplemented diet. The effect on membrane fluidity was obtained with pesticide concentration (about 10^{-7} M) that did not inhibit the enzyme.

2. Materials and methods

Male Sprague-Dawley rats grown after weaning on basic diet supplemented with 5% corn oil or lard were used in order to obtain erythrocyte membranes exhibiting low or high fatty acid fluidity respectively [9]. Blood samples were taken after 15 weeks by heart puncture and centrifuged 5 min at 600 × g. The plasma and the intermediate layer of white cells were discarded. The erythrocyte suspension was washed three times with a solution containing 155 mM NaCl, 2 mM sodium phosphate buffer, pH 7.4 mM and 10 mM glucose and finally adjusted to the original hematocrit in the same solution. The red cell suspension was stored at 4°C and used within 3 days. Erythrocyte suspensions which were stored for more than 24 h received and additional washing before use.

Acetylcholinesterase activity was measured by the

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method of Ellman et al. [10]. The standard incubation mixture, prepared at 0–4°C, contained 155 mM sodium phosphate buffer, pH 8.0, erythrocyte suspension (dilution 1:350), 0.5 mM acetylcholine iodide (ATC) in a final volume of 3.5 ml. The reaction was started by incubating two sets of tubes at 30°C, one set of tubes was placed in an ice bath after 5 min and the other one after 25 min of incubation. Each set of tubes was kept at 0–4°C for 3 min, then centrifuged at 600 × *g*. The optical density of the supernatants was determined at 435 nm with a Baush and Lomb colorimeter before and after the addition of 0.1 ml, 1.2 mM 5,5′-dithiobis-(2-Nitrobenzoic acid) (DTNB). The optical density before the addition of DTNB was substracted in each set of tubes in order to disregard hemolysis (not more than 1%) and the increase in optical density between 5 min and 25 min of incubation calculated.

Experiments with acetylcholinesterase from erythrocyte membrane and solubilized forms of the enzyme were performed according to previous reports [9,11] without including $MgCl_2$ in the incubation mixture. The specific activity and kinetic parameters of the enzyme were the same whether or nor Mg^{2+} was present in the incubation mixture.

When the organophosphorous effect was studied, before the addition of substrate, different enzymatic preparations were preincubated with pesticides, at specified final concentrations, for 15 min at 30°C. Then immediately placed in an ice bath (0–4°C) for 2 min and the substrate added. Controls were performed in the same way, then the reaction was continued as above indicated. The modification of the *n*-values by organophosphorous compounds was investigated by measuring the acetylcholinesterase activities under initial velocity conditions in the presence of F^- ranging from 0.5–5.0 mM. The *n*-values (Hill coefficient) for acetylcholinesterase were determined from the slopes in the Hill plots as previously described [9].

Parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothionate), Ethion (*O,O,O′,O′*-tetraethyl 5,5′-methylene-bis, phosphorodithioate) and Malathion (*O,O*-dimethyl 5-(1,2 dicarboxyethyl) phosphorodithioate) were kindly donated by FMC, Corporation, Agricultural Chemical Division, Middle Port, NY 14105, were dissolved first in 98% ethanol then 155 mM sodium phosphate, pH 8.0, immediately before use. When diluted in buffer to the final concentrations indicated in enzymatic assays, ethanol was present at 0.05% or lower, which had no effect on the kinetic parameters of the acetylcholinesterase.

ATC and DTNB were purchased from Sigma Chemical Co., St. Louis were dissolved in water and 155 mM sodium phosphate buffer, pH 8.0, respectively.

3. Results and discussion

A high correlation ($r = 0.90$) was reported between the *n*-value for the allosteric inhibition by F^- of erythrocyte membrane-bound acetylcholinesterase from rats fed with a different fat-supplemented diet and the *n*-value of the membrane fluidity expressed as the ratio double-bond index/saturated fatty acids [9]. An increase in the fatty acid fluidity of the membrane was accompanied by parallel increase in the cooperativity of the enzyme. Furthermore, the role of fatty acid composition in the changes of *n*-values of this enzyme was confirmed by in vitro recombination experiments [12]. To study the pesticides' action on the membrane fluidity, two synthetic diets (not deficient in essential fatty acids and producing maximal differences in membrane fatty acid fluidity) were chosen: corn oil and lard supplemented diets [9].

The changes of *n*-values from 1.6–1.0 for the inhibition by F^- of acetylcholinesterase from rats fed a corn oil diet, when bound to membrane, in the presence of Malathion 5×10^{-7} M is show in fig.1A.

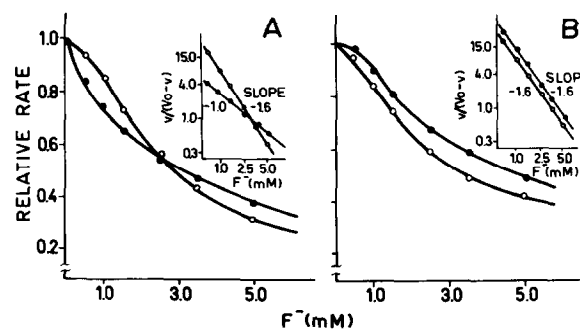

Fig.1. Inhibition by F^- of the membrane-bound (A) and soluble (B) acetylcholinesterase from rat, fed a corn oil diet in the absence (o–o) or in the presence (•–•) of 5×10^{-7} M of Malathion. Inset Hill plots.

Table 1
Effect of Malathion, Ethion and Parathion on the n-values for the inhibition by F^- of membrane-bound or soluble acetylcholinesterase from rats fed a corn oil or lard diet

Diet[a]	Enzymatic prepn	Control	Malathion (n-Values[b])	Parathion	Ethion
Corn oil	Red cells	1.55	0.90	0.90	1.05
	Membrane	1.53	0.90	0.90	–
	Soluble	1.60	1.63	1.55	1.53
Lard	Red cells	0.90	0.90	0.93	–

[a] Organophosphorous compounds were used at concentration of 5×10^{-7} M
[b] The data are the average of at least three independent experiments which did not differ by more than 0.1 in n-values

The n-values remained unchanged for the enzyme in the soluble form ($n = 1.6$) (fig.1B). Table 1 summarizes the results obtained with the enzyme bound to membrane or bound to intact whole red cells or soluble form. Similar behavior for Parathion, Ethion and Malathion were found. These compounds did not modify the n-values of membrane-bound enzymes from rats fed a lard supplemented diet.

The above mentioned organophophorous compounds decreased only the allosteric behavior of the enzyme bound to membrane with high double-bond index/saturated fatty acid ratio. The positive relationship between the n-values and membrane fatty acid fluidity [9] plus the fact that the soluble form of acetylcholinesterase was not affected, suggested that these compounds decreased the membrane fluidity.

Figure 2 shows that Malathion and Parathion decreased the membrane fluidity (evaluated through

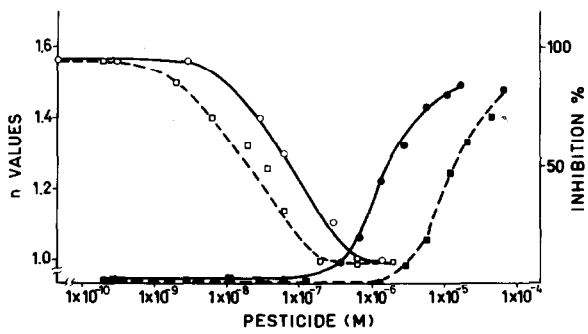

Fig.2. n-Values (○, □) and percent of inhibition (●, ■) of the erythrocyte acetylcholinesterase as function of Malathion (- - -) of Parathion (——) concentrations.

n-values) at concentration levels that did not inhibit the acetylcholinesterase activity. Besides, it is interesting to note that Malathion modified the membrane fluidity at a lower concentration than Parathion, but a higher concentration was necessary for the inhibition of the acetylcholinesterase. Half-maximal effect for changes of n- and enzymatic activity-values were obtained with 1.5×10^{-8} M and 2×10^{-5} M of Malathion and 6×10^{-8} M and 2×10^{-6} M of Parathion respectivily.

All results above indicated that the organophosphorous pesticides used in this work, besides inhibiting acetylcholinesterase at concentration levels of 10^{-7} M or higher, may produce changes in the membrane fluidity at concentrations of 10^{-7}–10^{-9} M. This effect was not demonstrated in membrane-bound acetylcholinesterase from rats fed a lard supplemented diet, but did not eliminate the possibility that the organophosphorous compounds were able to exert their effects in this membrane since, in this case, the cooperative transitions of the membrane-bound enzyme were at their minimal expression and by this probe no additional changes in membrane fluidity can be evaluated (for theoretical discussion see refs. [3,4]).

Inhibition of acetylcholinesterase 'in vitro' observed with organophosphorous pesticides used in this work may be due also to the presence of other organophosphate contaminants, such as paraoxon, malaoxon or the thiolo-form of parathio, S-ethyl parathion [2,13] (direct inhibitors). This fact does not negate the possibility that the former compounds or any of their metabolites may act at the membrane level.

The hydrophobic characteristics of the organophos-

phorous compounds and the nature of the substituent groups can probably explain the difference between the concentrations of Malathion and Parathion, necessary to obtain changes in the membrane fluidity, fig.2. The difference in the polar nature of the 'leaving group' (*S*-diethyl-succinate for Malathion and *p*-nitrophenyl for Parathion) could change the solubility or accessibility of the pesticides in the membrane. On the other hand, those pesticides at non-inhibiting concentrations, may produce phosphorylation of the membrane without attacking the enzyme, since the kinetic parameters of the soluble enzyme were not modified (see fig.1B) and in this way, change the allosteric behavior of the membrane-bound acetylcholinesterase, through changes in membrane fluidity.

We believe that the findings of this work may explain some phenomena that are not related in appearence to the neurotoxicity produced by inhibition of acetylcholinesterase and represents, to the best of our knowledge, the first report on relationship between pesticide action and membrane fluidity.

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