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A CONTRIBUTION TO THE MECHANISM OF ACTION OF SAD-128

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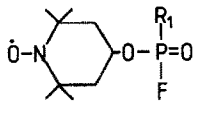
Abstract—SAD-128 was found to be an effective protector of acetylcholinesterase against inhibition by soman, due to its ability to function as a reversible inhibitor and allosteric modifier of the AChE active site. It also attenuated aging of the soman-inhibited enzyme. In order to study the connection between some of these effects of SAD-128 and structural changes in acetylcholinesterase and/or the membrane to which the enzyme is bound, the influences of SAD-128 on the EPR spectra of the spin labelled enzyme and of the membrane were studied under various conditions and the results correlated with the kinetic parameters. SAD-128 increases the fluidity of human erythrocyte membranes but not that of the *Torpedo marmorata* electric organ. Similarly, the binding properties of membrane acetylcholinesterase for SAD-128, expressed in terms of the Hill coefficient, differ for the two preparations. Some structural changes in the enzyme active site were also observed in the presence of SAD-128. The high protective effect of SAD-128 against AChE inhibition was confirmed by the EPR method regardless of the organophosphorus inhibitor tested. On the other hand, the effect of SAD-128 on the retardation of irreversible inhibition of the enzyme essentially depends on the inhibitor used. From present results it can be concluded that the protective effects of SAD-128 against inhibition of *m*-AChE are related to the structural changes of the active site and can be additionally moderated by the microviscosity changes of the membrane.

In addition to oxime derivatives of pyridinium salts, certain other compounds, which do not possess the oxime moiety and do not reactivate inhibited acetylcholinesterase (AChE; EC 3.1.1.7), can act as effective antidotes in the treatment of organophosphorus compound intoxication [1]. One such compound is SAD-128 [1, 2]; it is beneficial in soman poisoning when administered either prophylactically or therapeutically. It was suggested that SAD-128 protects against inhibition by soman due to its ability to function as a reversible inhibitor, and as an allosteric ligand modifier of inhibited AChE by decreasing the rate of aging of the soman–AChE complex, thus prolonging the susceptibility of the complex for reactivation by other reactivating agents [3–5].

The aim of the present work is to shed some light on the mechanism of action of SAD-128. Its influence on the active site of AChE as well as on the fluidity of the membrane, to which the enzyme is bound [6], was investigated by EPR and enzyme kinetic measurements. In order to study the allosteric, inhibitory and retardation properties of SAD-128 spin labelled organophosphorus compounds with general structural formula:



$R_1 = C_n H_{2n+1}$
$= OC_n H_{2n+1}$
$n = 1, 2, 3$

were used as irreversible inhibitors of *m*-AChE [7]. Their binding to the active site of *m*-AChE was investigated in the presence and absence of SAD-128. From the differences in the EPR spectral intensity it was possible to gain some data about the inhibitory and retardation influences of SAD-128 on the binding of the spin labelled inhibitors to the active site of *m*-AChE. Namely, the binding of spin labelled inhibitors to the enzyme active site is reflected in the EPR spectral intensity increase and in the corresponding decrease of the enzyme activity. On the other hand, from the line-shape changes of the EPR spectra structural alterations of the enzyme active site, which could be connected with the allosteric effect of SAD-128, were studied [8].

AChE is attached to the lipid environment of the membrane via a link with inositol phosphate and diacylglycerol [6]. Its structure and activity could be influenced by membrane fluidity changes and can be measured by EPR. Using the spin probe, which is well dissolved in the membranes, the possible alterations in the membrane fluidity under the action of SAD-128 were investigated and the results compared with the kinetic properties of the enzyme.

MATERIALS AND METHODS

Materials. The following chemicals were used: *Protective substance:* SAD-128 (1,1′-oxydimethylene bis(4-tert-butylpyridinium chloride) from the Faculty of Science, University of Zagreb; purity of the compound > 99%. *Spin labelled organophosphorus compounds:* MeSL (1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl methylphosphonofluoridate, EtSL (1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl ethylphosphonofluoridate, PrSL (1-oxyl,2,2,6,6-tetramethyl-4-piperidinyl propylphosphonofluoridate,

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MeOSL (1-oxyl,2,2,6,6-tetramethyl-4-piperidinyl methylphosphorofluoridate), EtOSL (1-oxyl,2,2,6,6-tetramethyl-4-piperidinyl ethylphosphorofluoridate), PrOSL (1-oxyl,2,2,6,6-tetramethyl-4-piperidinyl propylphosphorofluoridate). *Membrane spin probe:* MeFASL (methylester of 5-doxylpalmitate).

All spin labelled substances were synthesized by Prof. S. Pečar, Department of Pharmacy, Faculty of Natural Sciences and Technology, E. Kardelj University of Ljubljana, and were chromatographically pure.

Modified elasmobranch Ringer solution [9] in which bicarbonate buffer was replaced by 2 mM Tris–HCl (elasmobranch medium), pH 7.4, and saline buffered by Tris–HCl (saline), pH 7.4, were prepared from chemicals of analytical grade.

Torpedo marmorata electric organs were removed from fishes of either sex after anaesthetizing them in sea water of 0° for 1 hr. For the experiments on fresh tissue the tissue was kept at 0° in oxygenated elasmobranch medium for not longer than 24 hr; for the measurements on frozen tissue, the tissue was frozen immediately after excision and kept at −20°.

Fresh human blood was obtained from Blood Transfusion Institute of Slovenia.

Membrane preparations. The electric organ (10 g) of *Torpedo marmorata*, either frozen or fresh, was sheared in 30 mL of elasmobranch medium, pH 7.7; after shearing the pH was immediately adjusted with 0.1 mM NaOH to 7.4 and the preparation centrifuged for 30 min at 40,000 *g* at 0° (for details see Ref. 10).

Human blood was centrifuged at room temperature for 10 min at 3000 *g* and the pellet with erythrocytes was washed by saline three times at the same conditions. Finally, the erythrocytes were resuspended in saline to restore the original blood volume.

Spin labelling of the membranes. The membranes of *Torpedo marmorata* electric organ or of human erythrocytes were spin labelled with 10 μM MeFASL, which dissolves primarily in the phospholipid bilayer of the membranes. Seventy milligrams of the sediment of *Torpedo marmorata* electric organ suspended in 2 mL of elasmobranch medium, or a suspension of human erythrocytes in saline (1:1 v/v) were incubated with MeFASL for 30 min with continuous stirring at room temperature and centrifuged [11]. Ten microlitres of the corresponding medium (control) or 10 μL of the medium containing 10 μM of SAD-128 were added to 100 μL of the sediment.

For EPR measurements about 20 μL of the suspension of the electric organ membrane particles or of the erythrocyte suspension were placed in a capillary tube. The spectra were recorded on a Varian E-9 X-band EPR spectrometer: microwave power was 20 mW, modulation frequency 100 kHz, modulation amplitude 0.2 mT, and the temperature range from 25 to 40°.

Spin labelling of the m-*AChE active site of the electric organ.* One hundred milligrams of the sediment of the membrane preparation of the *Torpedo marmorata* electric organ was resuspended in 15 mL of elasmobranch medium, pH 7.4, and incubated with continuous stirring at room temperature for 30 min with one of the spin labelled organophosphorus compounds in a final concentration of 5 μM. After incubation, the suspension was diluted with 150 mL of elasmobranch medium, incubated for another 20 min and then washed by centrifugation at 40,000 *g* for 30 min at 0°. The washing procedure was repeated three times with 180 mL and finally with 50 mL of elasmobranch medium. Under these conditions selective and irreversible binding of the spin label to the active site of *m*-AChE was obtained; for details see Šentjurc *et al.* [7].

For EPR measurements about 50 μL of the enzyme preparation were placed in a quartz tissue cell and measured at room temperature on a Varian E-9 X-band spectrometer.

Spin labelling of the m-*AChE active site of the electric organ in the presence of SAD-128.* In order to check the protective effect of SAD-128 against irreversible inhibition of *m*-AChE by spin labelled organophosphorus compounds, the above spin labelling procedure was changed as follows: 5 min before addition of the spin label, SAD-128 (final concentration 1 mM) was added to the suspension of the membrane preparation and after incubation with the spin label, the mixture was diluted with 150 mL of elasmobranch medium containing 1 mM SAD-128, incubated for 20 min and washed as described previously.

In order to test the possible retardation of the irreversible inhibition of *m*-AChE by organophosphorus compounds caused by SAD-128, the spin labelling procedure was reversed: after incubation first with the spin labelled organophosphorus compound, the enzyme preparation was diluted with elasmobranch medium containing 1 mM SAD-128, incubated for 20 min and washed as already described.

EPR spectral intensity was measured relative to the strong pitch standard in a dual sample cavity. Since the line shape of the spectra did not change, the intensity measurements were performed by measuring the peak height of the middle EPR spectral line (h_0 in Fig. 2).

In order to examine the possible allosteric influence of SAD-128 on *m*-AChE, in some experiments 1 mM SAD-128 was added to the last washing medium. In parallel experiments 5 μL SAD-128 solution (10 mM) was added to 50 μL of the sediment of the *Torpedo marmorata* electric organ. These experiments were performed only on fresh tissues.

Denaturation of the spin labelled active site of m-*AChE of the electric organ by urea.* After spin labelling of *m*-AChE by one of the spin labelled organophosphorus compounds, 60 mL of elasmobranch medium containing 1 mM SAD-128 was added for 20 min; then the unreacted and the detached spin label was washed away using elasmobranch medium with 2, 4 or 8 M urea and 1 mM SAD-128. In control experiments elasmobranch medium with the same urea concentration was used, but without SAD-128.

Kinetic studies. The pellet of 5 g fresh tissue of *Torpedo marmorata* electric organ was resuspended in 30 mL elasmobranch medium, pH 7.4. For enzyme activity measurements 20 μL of the above suspension

in 15 mL elasmobranch medium, or 200 μL of erythrocyte suspension in 15 mL saline were used. The enzyme activity was measured by pH static titration at pH 7.4 and 25° in the presence of various concentrations of SAD-128 and constant ACh concentration (1 mM) (for details see Ref. 10). The binding properties of *m*-AChE for SAD-128 were determined according to Monod *et al.* [12]; they are described in terms of the Hill coefficient (n_H).

The enzyme activity of AChE was also measured after spin labelling of the active site of the enzyme of the *Torpedo marmorata* electric organ in the presence or absence of SAD-128.

RESULTS

Effect of SAD-128 on membrane acetylcholinesterase activity

Inhibition of enzyme activity at pH 7.4 is concentration dependent (Fig. 1). The reduction of the enzyme activity by SAD-128 for both the electric organ and erythrocytes is a linear function of concentration on a logarithmic plot over the whole concentration range studied. The concentration of SAD-128 required to inhibit 50% of AChE (IC_{50}) was found to be $(1.10 \pm 0.09) \times 10^{-4}$ M for the erythrocyte *m*-AChE and $(1.4 \pm 0.1) \times 10^{-4}$ M for *Torpedo m*-AChE. There is also a statistically significant difference between the enzymes in their n_H values: for *m*-AChE of human erythrocytes n_H is 0.80 ± 0.03 (N = 8) and for *Torpedo* electric organ n_H is 0.99 ± 0.05 (N = 11); each value is the mean ± SE.

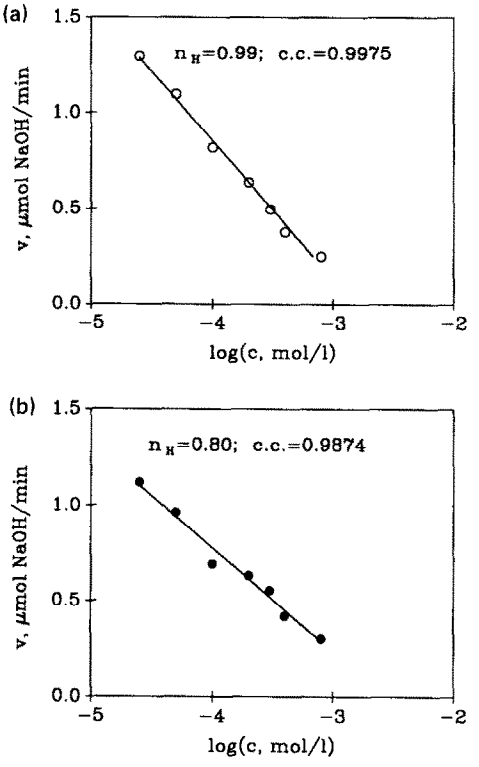

Fig. 1. ACh hydrolysis (1 mM) catalysed by membrane bound acetylcholinesterase in the presence of various concentrations of the non-oxime bispyridinium compound SAD-128. (a) *Torpedo marmorata* electric organ. (b) Human erythrocytes. Data points represent individual measurements of the representative experiment. The enzyme activity was measured at pH 7.4 and 25° for 5 min. The numerical values presented on the top of the figures are the Hill coefficients (n_H) with the corresponding correlation coefficient (c.c.).

Interaction of SAD-128 with cell membranes

Information about the interaction of SAD-128 with cell membranes was obtained from the measurements of the correlation time τ_c of the spin probe MeFASL incorporated into the membrane of the *Torpedo marmorata* electric organ or human erythrocytes at various temperatures. The correlation time gives information about the rotational motions of the phospholipid alkyl chains in the membranes. The shorter is τ_c, the more fluid, or in other words, the less viscous is the membrane. An empirical value of τ_c was determined from the EPR spectra according to the relation:

$$\tau_c = K\Delta H_0(\sqrt{h_0/h_{-1}} - 1)$$

where ΔH_0, h_0 and h_{-1} are parameters which can be measured from the EPR spectra: ΔH_0 is the linewidth of the middle line of the EPR spectra, while h_0 and h_{-1} are the amplitudes of the mid- and low-field line as is noted in Fig. 2, while K is a constant typical of the spin probe used [13]. This relation is valid only for fast isotropic motion of the spin probe and was used in our experiment as a rough approximation to follow the relative changes in the dynamics of spin label motion. The results are presented in Table 1. In the presence of 1 mM SAD-128 a significant decrease in τ_c was observed only for erythrocyte membranes at temperatures below 30° ($P < 0.05$, using the matched pair *t*-statistic). From these experiments it can be concluded that SAD-128 increases the membrane fluidity of human erythrocytes, but has no significant effect on the membranes of the *Torpedo marmorata* electric organ.

Effect of SAD-128 on the retardation of irreversible inhibition of m-AChE by organophosphorus compounds and on protection against this inhibition

For the organophosphorus compound EtOSL the protective effect of SAD-128 on the enzyme activity, as well as its effect on retardation of irreversible inhibition, was obtained from the EPR spectral intensity differences under the action of SAD-128 (Fig. 3) as well as from the enzyme activity measurements. There is a significant difference in the intensity of the EPR spectra of the spin labelled organophosphorus compounds bound to the active site of *m*-AChE when SAD-128 is added to the preparation before or after the spin labelled organophosphorus compound. When SAD-128 is added before, the intensity is diminished by 80% in comparison to the intensity measured in the absence of SAD-128. These results are in good agreement with the enzyme activity measurements. Therefore, it can be concluded that the protection of *m*-AChE by SAD-128 was about 80% (N = 5). Similar results were obtained also for MeOSL, MeSL and EtSL; i.e. SAD-128 was a successful protective agent. When

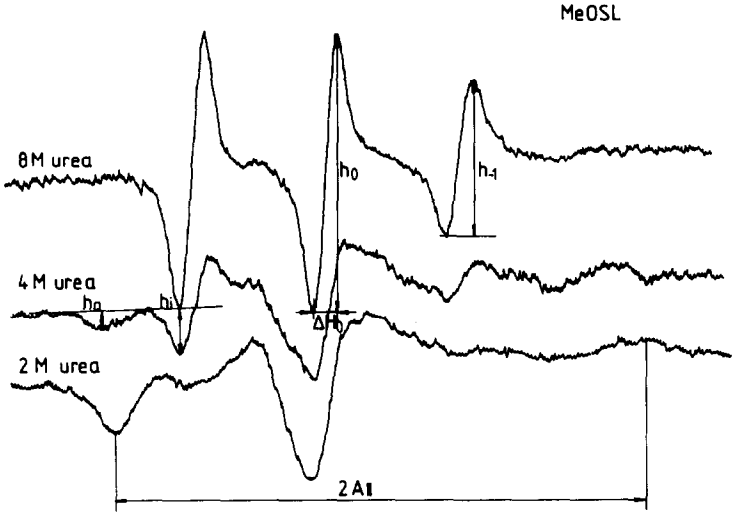

Fig. 2. EPR spectra of spin label, selectively bound to *m*-AChE of *Torpedo marmorata* electric organ after denaturation of the enzyme with different urea concentrations in the presence of SAD-128. All the EPR parameters used in the present study are indicated.

Table 1. Influence of the non-oxime bispyridinium compound SAD-128 (1 mM) on correlation time τ_c of the spin probe MeFASL incorporated into membranes of the *Torpedo marmorata* electric organ or human erythrocytes

Temperature (°C)	τ_c (nsec)			
	Electric organ		Erythrocytes	
	Control	SAD-128	Control	SAD-128
25	3.40 ± 0.14 (8)	3.49 ± 0.08 (8)	4.91 ± 0.14 (6)	4.53 ± 0.10 (6)
30	2.82 ± 0.10 (8)	2.85 ± 0.04 (8)	3.91 ± 0.24 (6)	3.56 ± 0.06 (6)
35	2.47 ± 0.08 (8)	2.44 ± 0.06 (8)	2.86 ± 0.04 (6)	2.95 ± 0.04 (6)
40	2.11 ± 0.12 (8)	2.08 ± 0.09 (8)	2.63 ± 0.11 (4)	2.57 ± 0.05 (4)

Experimental details for membrane preparations are described in Materials and Methods. Results are the mean values ± SE; number of experiments in each group is given in parentheses.

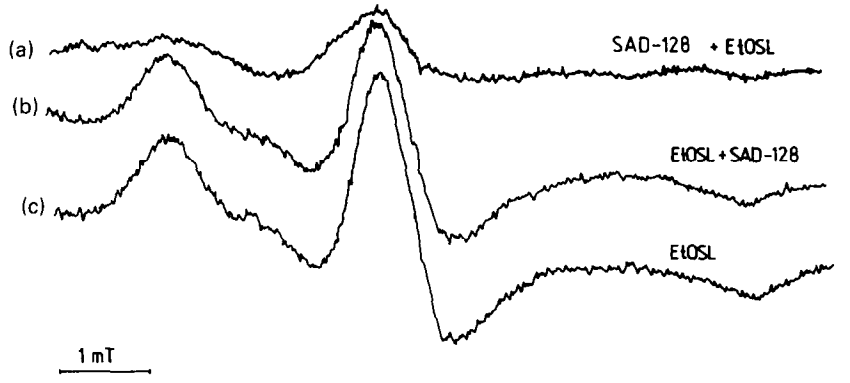

Fig. 3. The influence of SAD-128 on the intensity of EPR spectra of the spin label EtOSL selectively bound to the membrane acetylcholinesterase of the electric organ. (a) Protection: the enzyme preparation was preincubated for 5 min with SAD-128 before spin labelling. (b) Retardation of irreversible inhibition: the enzyme preparation was incubated with spin label for 30 min and then the incubation medium diluted with a medium containing SAD-128 to a final concentration 1 mM, and incubated for another 20 min. (c) Control: the enzyme preparation was incubated with 5 μM spin label for 30 min at room temperature with continuous stirring.

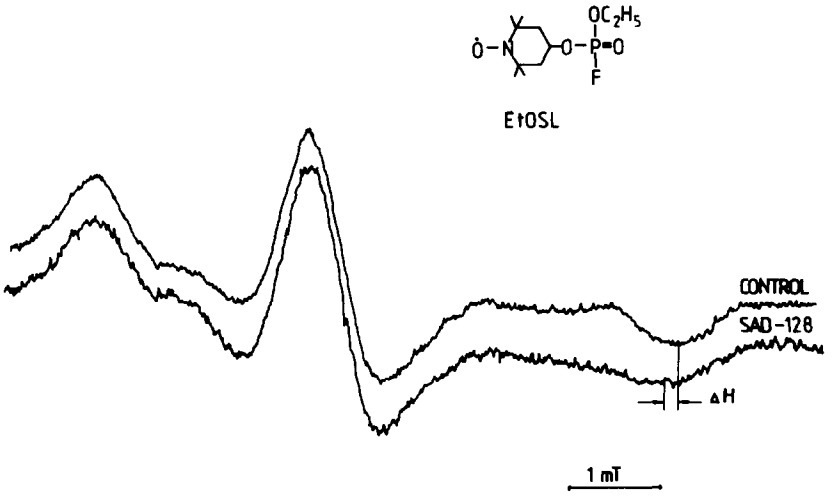

Fig. 4. EPR spectra of spin label EtOSL, selectively bound to the *m*-AChE of *Torpedo marmorata* electric organ in the presence and absence of SAD-128. After recording the control spectrum, SAD-128 (1 mM) was added to the sample at a final concentration of about 1 mM.

SAD-128 was added 30 min after labelling about 30% (N = 6) of EtOSL was washed out from the active site of AChE, as determined from the intensity decrease of the EPR spectra and from the enzyme activity measurements; for MeOSL, MeSL and EtSL no significant changes were observed. The stoichiometry of the number of active serines inhibited by the spin labelled organophosphorus compounds to the number of selectively bound spin labelled molecules [7] also indicates that under these conditions no aging process took place, which would have resulted in a loss of the alkyl group bearing the nitroxide radical.

Structural changes of the active site of m-*AChE of the electric organ induced by SAD-*128

In order to determine the possible structural changes of the active site of *m*-AChE, EPR spectra of the spin labelled organophosphorus compound EtOSL were measured in the absence and in the presence of 1 mM SAD-128 (Fig. 4). In six experiments SAD-128 was added to the final elasmobranch medium, while in 21 experiments it was added to the sediment after recording the control. From the EPR spectra the maximal hyperfine splitting $2A_{\|}$ (defined in Fig. 2), which is directly related to the rotational freedom of the piperidine ring of the spin label bound to the *m*-AChE active site, was determined. In both sets of experiments a decrease of maximal hyperfine splitting $2A_{\|}$ was found; the difference in the hyperfine splitting $\Delta 2A_{\|} = 0.11 \pm 0.04$ mT (mean ± SE, N = 27, $P < 0.02$). This means that after the action of SAD-128 the rotational freedom of the piperidine ring of EtOSL, which is selectively bound to the enzyme active site, was increased, indicating that under the influence of SAD-128 some stearic hindrances in the active site were diminished. With all other organophosphorus compounds measured in the presence of SAD-128, no changes in $2A_{\|}$ were observed (number of independent experiments ≥ 5).

Denaturation of m-*AChE active site of the electric organ in the presence of SAD-*128

EPR spectra lineshape changes, after denaturation of the enzyme active site by urea in the presence or absence of SAD-128, are presented in Fig. 2 and Table 2. After treatment with 2 and 4 M urea in elasmobranch medium, the spectra are the superimposition of at least two populations of spin labelled molecules with different motional freedom. Denaturation was characterized by the ratio h_i/h_a (Fig. 2) which is related to the number of less restricted (h_i) and more restricted motions (h_a) of the spin label molecules, selectively bound to the active site of various degrees denaturated enzyme. The fraction with higher motional freedom and consequently the h_i/h_a ratio, increases with increasing urea concentration. In 8 M urea only the fraction with high motional freedom is observable. From the present results no significant influence of SAD-128 on denaturation by urea is observable.

DISCUSSION

Measurements of enzyme activity in the presence of SAD-128 confirm that SAD-128 is an inhibitor of *m*-AChE, as already indicated in previous studies [3]. However, its effect differs in regard to the two membrane bound enzymes studied. In view of the allosteric nature of *m*-AChE [14, 15], the different n_H values measured could be interpreted in terms of different conformations of the two enzymes. The $n_H \simeq 1$ obtained for *m*-AChE of the *Torpedo* electric organ indicates that the binding of SAD-128 is non-cooperative, which points to the existence of allosterically independent subunits of *m*-AChE. On the other hand, the $n_H \simeq 0.80$ obtained with erythrocyte *m*-AChE indicates that the binding of SAD-128 is negatively cooperative, which might be due to the existence of allosterically interdependent subunits of this *m*-AChE.

Table 2. The influence of urea on the ratio h_i/h_a of EPR spectra of spin labels bound to the *m*-AChE active site of the electric organ

	h_i/h_a*			
	MeOSL		EtOSL	
Concentration of urea (M)	in presence of SAD-128	in absence of SAD-128	in presence of SAD-128	in absence of SAD-128
0.38†	0.12 ± 0.05	0.14 ± 0.05	0.2 ± 0.1	0.05 ± 0.02
2	0.30 ± 0.08	0.09 ± 0.06	0.4 ± 0.1	0.4 ± 0.1
4	1.6 ± 0.4	1.7 ± 0.6	2.2 ± 1.0	3.3 ± 0.9

Values are mean ± SE of five samples in each case.
* See Fig. 2.
† In elasmobranch medium.

Since *m*-AChE is attached to the lipid environment, the difference in n_H might also reflect the observed different influences of SAD-128 on the membrane microviscosity in the two samples (Table 1), and this may be relevant for the magnitude of the interaction between protein and lipid in the membrane [16]. The portion of the AChE molecule inserted into the lipid bilayer is distant from the active site [17], and thus we might be justified in suggesting that the peripheral site of AChE consisting of a matrix of partially overlapping loci [18] can be modified by the microviscosity changes. This could be consequently reflected in the structure of the active site [18]. Therefore, when AChE is inserted in the membranes with different properties, the same ligand could induce different conformational changes in its active site.

For EPR studies of the *m*-AChE active site, only samples of *Torpedo* electric organ can be used because only this preparation contains an adequate concentration of active serines for binding the spin labelled organophosphorus compounds in a volume suitable for EPR measurements in the tissue cell (*ca.* 50 μL or 0.05 g). In order to get a reliable EPR signal in this volume, there should be about 10^{14} spin label molecules attached to the sample; in an erythrocyte ghost suspension this quantity of binding sites was contained in 15 mL or above [19].

In agreement with previous reports based on different methodology [2, 3], the strongly protective effect of SAD-128 against AChE inhibition was confirmed using the EPR method, regardless of the irreversible inhibitor tested. However, the effect of SAD-128 on retardation of irreversible inhibition of *m*-AChE by spin labelled organophosphorus compounds depends essentially on the inhibitor used. Namely, a significant effect was observed when the enzyme was incubated with EtOSL, but not with MeOSL. Similarly, the structural changes in the inhibited active site of *m*-AChE induced by SAD-128 were observed only when the inhibitor was EtOSL. The changes in the line-shape of the EPR spectra in the presence of SAD-128 are manifested only in a small decrease of $2A_{\|}$ and not in an overall line-shape alteration, which is typical for denaturation of the enzyme [7]. Therefore, with respect to the pocket-like structure of the active site of *m*-AChE [8, 20], the observed changes in the microgeography of the active site are attributed to the allosteric effects of SAD-128.

The fact that the induced allosteric changes of the active site of *m*-AChE were found only when the enzyme was labelled with EtOSL implies some specific properties of this spin labelled phosphorofluoridate. This specific property of EtOSL was also expressed in QSAR analysis of 27 spin labelled organophosphorus compounds by the use of the structure similarity method, which was done in order to predict biomolecular rate constants for four additional fluorophosphonic acid derivatives. The predicted and measured values were in perfect agreement for MeOSL and PrOSL, while for EtOSL the measured value was approximately 20 times lower than the calculated one [21]. It seems that the three-dimensional structure of EtOSL impedes its binding to the enzyme active site; however, when it is bound, it is more susceptible to various configurational changes of the active site than the other organophosphorus labels used in the present study.

Since the local structural perturbations of the active center can notably change the apparent conformational stability of this center [22], urea denaturation of the spin labelled enzyme was followed by EPR. Although structural changes in the active site induced by SAD-128 were found, its possible influence on the denaturation process caused by urea was not detected by EPR. Namely, in the EPR spectra no significant differences of the ratio of h_i/h_a were measured in the presence or absence of SAD-128 (Table 2).

The present results of kinetic and EPR studies of *m*-AChE confirm the action of SAD-128 as an inhibitor and allosteric modifier, as well as its protective effect against irreversible inhibition of *m*-AChE by organophosphorus compounds [3–5]. Further, it can also be concluded that the allosteric influence of SAD-128 on the active site of the enzyme is connected with the structural changes of the active site and that these can be additionally moderated by microviscosity changes of the membrane. As already mentioned, the structural changes of the *m*-AChE active site by SAD-128 were observed only with EtOSL. At the same time this is the only spin labelled organophosphorus inhibitor for which retardation of the irreversible inhibition was observed under the

influence of SAD-128. This indicates that this inhibitor is specifically wedged into the active center, as was also confirmed by the lower biomolecular rate constant of EtOSL than for the other organophosphorus inhibitors investigated in this study [21].

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