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THE INFLUENCE OF SOMAN SIMULATOR ON REACTIVATION BY HI-6 OF SOMAN-INHIBITED ACETYLCHOLINESTERASE IN PREPARATIONS OF RAT AND HUMAN SKELETAL MUSCLE

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Abstract—The aim of our study was to elucidate the phenomenon called "soman depot". Our investigations were focused on the depot formed in the skeletal muscle and on the effects of 1,2,2-trimethylpropyl dimethylphosphonate (PDP), a reported blocker of soman depot formation. The following questions were addressed: (1) how much of acetylcholinesterase (EC 3.1.1.7, AChE) activity can additionally be recovered by Hagedorn bispiridinium oxime reactivator 2-hydroxyimino-methyl-pyridinium-1-methyl-4'-carbamoyl-pyridinium-1'-methylether dichloride monohydrate (HI-6) in the skeletal muscle preparations if they are pretreated by PDP prior to incubation in soman (1,2,2-trimethylpropyl methylphosphonofluoridate)? (2) Is this effect uniform along the muscle fibre or different in the endplate in comparison to the endplate-free region? (3) Is the effect of PDP species specific, i.e. does it differ between rat and human muscle? (4) What are the molecular mechanisms of the effects of PDP? PDP pretreatment increased the reactivation of soman-inhibited AChE by HI-6 in both regions of rat skeletal muscle. This increase was smaller in human skeletal muscle. The PDP-mediated increase in HI-6 reactivation was most efficient in the endplate-rich region of rat diaphragm as demonstrated biochemically and histochemically, but it could not be explained by the blockade of soman depot alone since it was also observed at low soman concentrations, at which soman depot is not supposed to form. This PDP effect could be better explained by the direct interactions of PDP with AChE resulting in decreased AChE phosphorylation. Soman concentration-dependent increase in HI-6 reactivation by PDP, which was more efficient at a high than a low soman concentration and could therefore originate from blockade of soman depot, was observed in the endplate-free region of rat diaphragm. It was also found in human muscle but was again smaller in this species. According to our EPR study, solubilization of soman in the lipophilic cell membrane compartment can be excluded as a mechanism producing significant soman depot. In general, our results suggest a more complex mechanism of PDP action than reported previously.

After severe soman (1,2,2-trimethylpropyl methylphosphonofluoridate) poisoning (6–8 × LD_{50}), oxime therapy with 2-hydroxyimino-methyl-pyridinium-1-methyl-4'-carbamoyl-pyridinium-1'-methylether dichloride monohydrate (HI-6†) can lead to a rapid initial recovery of the animals, followed by deterioration of their conditions and their death in the subsequent 4–5 hr [1]. The generally accepted explanation of this phenomenon is that a part of the injected soman is stored and remains intact in a depot from where it is gradually released causing subsequent reinhibition of the acetylcholinesterase (AChE; EC 3.1.1.7) previously reactivated by the oxime. Important for this explanation were the experiments in which rat reintoxication was prevented by prophylactic treatment with so called soman simulators, i.e. compounds that resemble soman in chemical structure and lipophilicity but which have the fluorine atom replaced by a methyl group [1,2,2-trimethylpropyl dimethylphosphonate (pinacolyl dimethylphosphinate) PDP] or alkoxy group [2, 3]. Having such a structure simulators are unable to bind covalently to the serine in the AChE active center but can prevent soman storage; this is the current explanation of their beneficial effects. Pretreatment of soman-intoxicated rats with PDP prevents the failure of neuromuscular transmission in the rat diaphragm [4]. PDP pretreatment of rats also attenuates the inhibition of electric eel AChE injected into the rat blood 90 min after soman treatment or added into the medium into which muscles isolated from soman-intoxicated rats were placed [5]. The described PDP effect was observed only if PDP was given to rats 10 min before (better expressed) or immediately after soman (less expressed) but not if PDP was given 91 min after soman, i.e. 1 min after injection of electric eel AChE; in this case, AChE injected into the rat blood became even more inhibited. The effects of PDP were species specific: they were less expressed in guinea pigs and marmosets than in rats [5].

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† Abbreviations: AChE, acetylcholinesterase; HI-6, 2 - hydroxyimino - methyl - pyridinium - 1 - methyl - 4' - carbamoylpyridinium-1'-methylether dichloride monohydrate (Hagedorn bispyridinium oxime reactivator); iso-OMPA, tetraisopropylpyrophosphoramide (selective inhibitor of nonspecific cholinesterase); MeFASL, methyl-ester of 5-doxylpalmitate-spin label; PDP, 1,2,2-trimethylpropyl dimethylphosphonate (pinacolyl dimethylphosphinate) (soman simulator).

The aim of our study was to elucidate further the phenomenon of "soman depot" and especially to investigate whether and how PDP improves the reactivation by HI-6 of soman-inhibited AChE in skeletal muscle. The following questions were addressed: (1) How much of AChE activity can additionally be recovered by HI-6 in the skeletal muscle preparations by PDP pretreatment? (2) Is the effect of PDP pretreatment on the reactivation of AChE by HI-6 uniform along the muscle fibre or is there a difference between the endplate (predominantly extracellular AChE [6, 7]) and the endplate-free (predominantly intracellular AChE [6]) region? (3) Is the effect of PDP species specific, i.e. is there any difference between rat and human muscle in this respect? (4) What is the molecular mechanism underlying PDP effects?

The effects of PDP on the reactivation of soman-inhibited AChE by HI-6 in the skeletal muscle preparations were quantitatively determined by measuring per cent increase in AChE reactivation by HI-6 in soman-intoxicated muscle preparations pretreated with PDP. If PDP effects result from the elimination of soman depot, this per cent should correspond to the per cent of AChE inhibition by the depot soman in the non-pretreated preparations. Since a soman depot forms only at a high soman concentration [5, 8, 9], only those PDP effects which are found at high but not at low soman concentrations could be attributed to the elimination of soman depot. PDP effects which could also be observed at low soman concentrations must therefore be based on some alternative mechanism. In order to differentiate between depot blocking and other mechanisms of PDP actions, two different concentrations of soman were used when testing the effects of PDP: the low concentration was the smallest concentration of soman still able to give about 90% inhibition of control AChE activity after 5 min incubation; a very small amount of soman was expected to enter the depot at this concentration. The high concentration of soman was 10 times higher than the low and was, besides inhibiting AChE, expected to form soman depot. The concentration of PDP used for the pretreatment of muscle preparations was 10 times higher than the concentration of soman in all our experiments where PDP effects were tested; this soman/PDP ratio was also used by other authors for the prevention of soman depot [4]. Endplate-rich and endplate-free regions of rat diaphragm were studied separately. In order to verify biochemical measurements, histochemical localization of AChE in the motor endplates was followed under our experimental conditions. Experiments were carried out on both rat and human muscle preparations.

Molecular mechanisms of PDP effects were also investigated and two not yet studied possibilities were tested. First was the accumulation of soman and PDP in the cell membrane compartment: since both soman and PDP are relatively lipophilic [10] they might enter the membrane lipid compartment causing or preventing, respectively, in this way the formation of soman depot. Recent findings that soman influences membrane fluidity in the rat superior cervical ganglion [11] support such an assumption. In order to test this possibility an EPR study was carried out. Putative solubilization of soman and PDP in the cell membranes was determined by measuring the effects of both compounds on erythrocyte membrane fluidity. The second possible mechanism of PDP action tested in our experiments, i.e. the direct interaction of PDP with the soman phosphorylation of AChE has, at first sight, little to do with soman depot. However, some of the beneficial effects of PDP pretreatment could also be explained by the direct interactions of PDP with the soman phosphorylation of AChE. Having an almost identical chemical structure to that of soman, PDP might by non-covalent interactions interfere with the binding of soman to AChE causing in this way reduced AChE inhibition. A non-covalent organophosphate binding site was indeed discovered recently on the AChE in homogenates of mouse skeletal muscle, and binding of organophosphate to this site reduced phosphorylation at the AChE active site [12]. In order to find out whether PDP interferes with soman phosphorylation and HI-6 reactivation of the skeletal muscle AChE, per cent of soman inhibition of rat muscle AChE and HI-6 reactivation were determined in the presence of PDP. Very low soman concentrations were used in these experiments in order to prevent the formation of any soman depot. Homogenates of rat and human muscles were used instead of muscle preparations in order to have better-controlled experimental conditions.

MATERIALS AND METHODS

Materials. The following chemicals were used: racemic soman, PDP, HI-6, all provided by VTI, Belgrade; [1-^{14}C]acetylcholine chloride (0.98 GBq/mmol), Amersham (U.K.); iso-OMPA (tetraisopropylpyrophosphoramide), Koch Light (Colnbrook, U.K.); MeFASL (methylester of 5-doxylpalmitate): spin-labelled substance was synthesized by Prof. S. Pečar, Dept of Pharmacy, University of Ljubljana. Fresh human blood was obtained from the Blood Transfusion Institute of Slovenia. Electric eel AChE was from Worthington (NJ, U.S.A.).

Muscle preparations. Female Wistar strain albino rats weighing between 150 and 200 g were anaesthetized with ether and perfused with saline through the inferior vena cava. The diaphragm was removed with ribs attached and pinned out in a Sylgard-coated Petri dish. A strip of muscle about 3 mm wide from around the branches of the phrenic nerve (endplate-rich region) was cut and the remaining endplate-free region was isolated. Human muscle preparations were obtained at the Institute of Legal Medicine of the Medical faculty, Ljubljana. Intercostal muscles were isolated within 5 hr of death from the victims of traffic accidents. Longitudinal muscle preparations were obtained by cutting pieces of intercostal muscle at both ends from the ribs. Endplate-rich and endplate-free regions were not divided in human muscles. Preparations were of approximately the same thickness and weight (about 60 mg) as rat diaphragm preparations. After preparation, samples were weighed and frozen in liquid nitrogen until used.

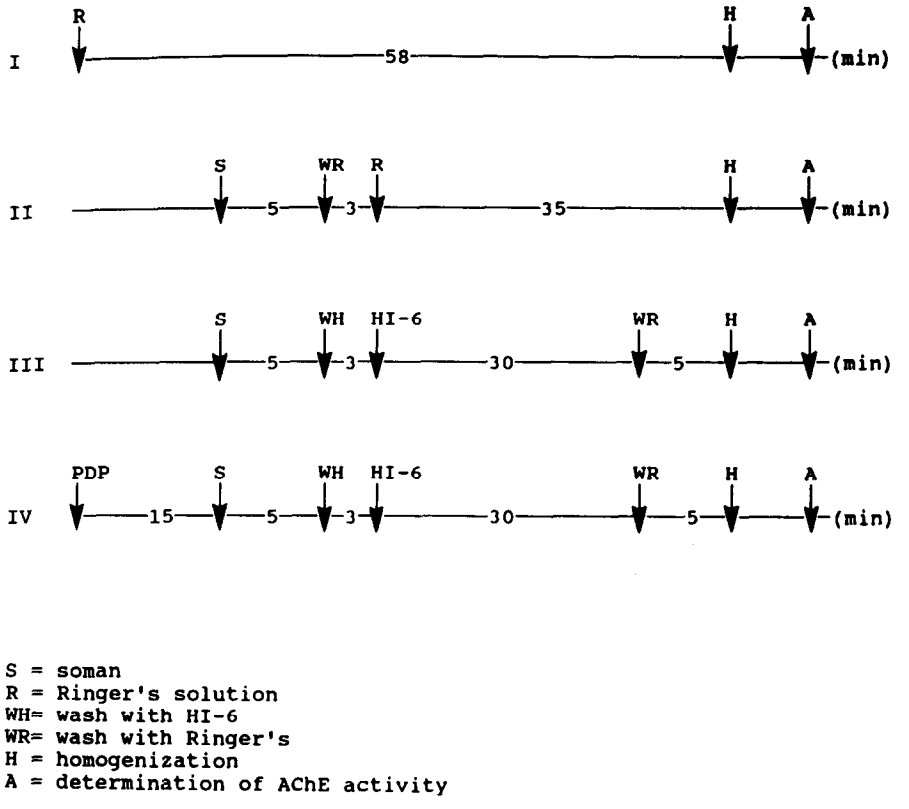

Fig. 1. Rat and human muscle preparations: general outline of the experimental approach. Four muscle preparations of the same weight and thickness were processed according to the four different but simultaneous procedures, numbered I, II, III and IV. (I) Control experiment, giving 100% activity; (II) AChE activity remaining after 5 min incubation with soman; (III) AChE activity reactivated during 30 min incubation in HI-6 after 5 min incubation in soman; (IV) the effect of PDP pretreatment on reactivation by HI-6: prior to soman exposure, muscle preparations were pretreated for 15 min in PDP at concentrations 10 times higher than that of soman. Control experiment testing the effects of PDP alone on the AChE activity in muscle preparations is described in the text. Final concentration of HI-6 was 0.5 mM.

Since rat muscle preparations were isolated fresh, while human muscle preparations were isolated up to 5 hr after death, we could not exclude the possibility that different results obtained from the two species reflect differences in autolytic damage rather than species-specific effects. Control experiments were therefore carried out in which rats were killed by ether anaesthesia and left dead for 5 hr before isolation of diaphragm muscles. These control experiments were carried out only on the preparations from the endplate-free region and at high soman concentrations, i.e. under conditions where the highest impact of tissue autolysis on the results could be expected. It is known that most of the AChE in the rat endplate-free region is located in the intracellular compartment, which is more affected by autolysis than the extracellular compartment, where most of the AChE from the endplate-rich region is located [6, 7]. High soman concentration was selected because PDP effects were expected to be expressed to a greater degree at this concentration. Results obtained in preparations isolated 5 hr after death were compared with the results from fresh tissue.

Rat and human muscle preparations: the effects of PDP on the reactivation of soman-inhibited AChE by HI-6. A general outline of the experiments is given in Fig. 1. The rationale for our experimental approach was to try to reproduce as much as possible the conditions (timing, concentrations) used by other authors in studies dealing with PDP and soman depot in skeletal muscle [4]. Media containing PDP, soman and/or HI-6 were prepared in phosphate Ringer's solution with the following composition (used in all our experiments): 138 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 20 mM phosphate buffer, pH 7.4. The final concentration of HI-6 was 0.5 mM throughout the experiments. Media were kept in a series of petri dishes at room temperature and muscle preparations were transferred from one medium to another according to the scheme. Prior to the determination of AChE activity, muscle samples were homogenized by hand-operated glass homogenizers kept on ice. A control experiment in which the effects of PDP alone on the AChE activity in the muscle preparation were tested was carried out in the same way as control experiment (Fig. 1) except that preparations (rat endplate-free region) were

first incubated for 20 min in PDP (18 μM), washed and then transferred into Ringer's solution where they were left for 38 instead of 58 min prior to homogenization. Homogenate concentrations were: 1 mg/10 μL for the preparations of the endplate-free region of rat diaphragm and for the preparations from human intercostals; 1 mg/20 μL for the endplate-rich region of rat diaphragm preparations. Homogenates were then preincubated with 10 μM iso-OMPA for 30 min on ice in order to inhibit non-specific cholinesterase activity. AChE activity was then determined radiometrically as described previously [13, 14]. Soman was applied at two different concentrations selected as described above. Under our conditions concentrations of soman selected for the treatment of rat muscle preparations were 0.18 μM (low) and 1.8 μM (high), respectively. Concentrations of soman selected for application to the human muscle preparations had to be lower and were 0.09 μM (low) and 0.36 μM (high), respectively. The PDP/soman concentration ratio was kept at 10/1 in all our experiments.

Histochemical demonstration of AChE in muscle preparations. Muscle preparations isolated from the endplate-rich region of rat diaphragm were pinned down in Sylgard-coated petri dishes. Media in the dishes were changed according to the scheme (Fig. 1). After the final wash with Ringer's solution, muscle preparations were fixed for 30 min in the fixative (2% paraformaldehyde and 1% glutaraldehyde). After washing, samples were cut to smaller pieces, which were stacked one upon another and frozen. Cross-sections (20 μm) were cut on a cryotome and mounted on glass slides. After 10 min incubation in modified histochemical medium of Koelle and Friedenwald [15], the slices were washed, cleared in glycerol, observed under a light microscope and photographed.

Homogenates of rat and human skeletal muscle: the effects of PDP on AChE activity, soman inhibition of AChE and reactivation of AChE by HI-6. Rat hemidiaphragms and human intercostals were isolated as described above, weighed and then frozen in liquid nitrogen until used. Muscle homogenates of both species (1.45 mg/10 μL of phosphate Ringer's solution) were prepared in hand-operated glass homogenizers kept on ice. Homogenate (H) (20 μL) was then incubated for 5 min at room temperature with the following combinations of phosphate Ringer, 0.2 nM soman, 2 mM PDP and 0.5 mM HI-6 (all concentrations are final): (A) H + 20 μL of Ringer's solution (control = 100% activity); (B) H + 20 μL soman (baseline inhibition of AChE); (C) H + 10 μL of soman + 10 μL of PDP (the effect of PDP on AChE phosphorylation by soman); (D) H + 10 μL of soman + 10 μL of HI-6 (reactivation of soman inhibited AChE by HI-6) and (E) H + 10 μL of soman + 5 μL of HI-6 + 5 μL of PDP (reactivation of soman inhibited AChE by HI-6 in the presence of PDP). After preincubation of mixtures A, B, C, D and E in 10 μM iso-OMPA (final concentration) for 30 min in order to block any remaining non-specific cholinesterase, all mixtures underwent radiometric determination of AChE activity as described before [13]. Radioactively labelled AChI (3 mM) was used for AChE activity determinations by TLC. The effect of PDP on the AChE activity in rat and human muscle homogenates was determined by incubation of 20 μL of homogenate with 20 μL of PDP (2 nM final concentration). In these experiments a 2 nM concentration of PDP was kept constant during preincubation with iso-OMPA and incubation with ACh. All other conditions were the same as described for mixtures A–E.

The effect of PDP on the activity of electric eel AChE. The effects of PDP (at 2.4 and 1.2 mM final concentrations) on the electric eel AChE activity solubilized in phosphate Ringer's solution were determined using standard Ellman's procedure [16]. Rates of acetylthiocholine hydrolysis were determined in the substrate concentration range from 40 μM to 10 mM.

EPR measurements. Experiments were carried out on both rat and human erythrocytes. Erythrocytes were spin labelled with 10 μM methyl ester of 5-doxylpalmitate (MeFASL, Fig. 3), which dissolves primarily in the phospholipid bilayer of the membranes. A suspension of erythrocytes in saline (1:1 v/v) was incubated at room temperature and centrifuged as described [17]. After labelling the cells were incubated in 100 μL volume either with saline or with the following compounds or their combinations dissolved in saline: (1) 10 μM soman, (2) 10 μM PDP, (3) 100 μM PDP, (4) 10 μM soman + 100 μM PDP (all concentrations are final). These concentrations were selected after initial experiments at low concentrations of PDP at which no conclusive effects were observed. For EPR measurements about 20 μL of the erythrocyte suspension were placed in a capillary tube. EPR measurements were performed on a Varian E-9 X-band EPR spectrometer at the Institute Jožef Stefan in Ljubljana: microwave power was 10 mW, modulation frequency 100 kHz, modulation amplitude 1 mT. Measurements were performed at different temperatures ranging from 20° to 41°. From EPR spectra measured at 20° or 25°, the maximal hyperfine splitting $2A_{//}$ (Fig. 3) which is directly related to the order parameter was determined. From the EPR spectra measured at higher temperatures, the empirical correlation time τ_c was calculated using the relationship:

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

Δ, H_0, h_0 and h_{-1} are parameters, which can be measured from the EPR spectra and are defined in Fig. 3, while K is a constant typical for the spin label probe used; see Ref. 18 for details.

RESULTS

Rat and human skeletal muscle preparations: the effects of PDP on the reactivation of soman-inhibited AChE by HI-6

A PDP-dependent increase in AChE reactivation by HI-6 was found in all our experiments (Tables 1 and 2). It was greater in rat muscle (both regions) than in human muscle (Table 2). In the control experiments (N = 4) in which rat muscle preparations (endplate-free region) isolated 5 hr after death were used instead of fresh tissue, reactivation by HI-6

Table 1. Whole muscle preparations: the effects of PDP on AChE inhibition by soman and on the subsequent reactivation of inhibited AChE by HI-6

	I (%)	II (%)	III (%)	IV (%)
High soman concentration				
REPR	100	3.5 ± 3.8	25.4 ± 4.6	52.6 ± 14.1
REPF	100	3.7 ± 3.7	7.6 ± 2.3	26.2 ± 9.2
HM	100	3.9 ± 2.7	17.7 ± 8.2	28.4 ± 12.0
Low soman concentration				
REPR	100	3.4 ± 3.3	36.0 ± 11.8	66.7 ± 14.3
REPF	100	2.7 ± 2.2	24.5 ± 5.8	33.1 ± 5.3
HM	100	16.7 ± 6.5	28.5 ± 11.2	35.1 ± 15.4

High soman concentration: 1.8 and 0.36 μM for rat and human muscle preparations, respectively; low soman concentration: 0.18 and 0.09 μM for rat and human muscle preparations, respectively.

REPR, rat endplate-rich region; REPF, rat endplate-free region; HM, human muscle.

PDP was used at a concentration 10 times higher than that of soman throughout the experiments; HI-6 concentration was 0.5 mM.

Procedures I–IV are as described in Fig. 1. Values under II–IV are percentages of control (I) AChE activity (mean ± SD; N = 5).

All values differ significantly from each other in all three different groups of muscle preparations tested ($P < 0.05$). The only exceptions are the non-significant difference ($P < 0.05$) between II and III in REPF (high soman concn) and between III and IV in HM (low soman concn). Statistical analysis of variance or equivalent non-parametric Kruskal–Wallis test followed by Bonferroni comparison test was used.

(III − II) was higher (17.9 vs 3.9% in fresh tissue) and so was HI-6 reactivation in the PDP-pretreated preparations (IV–II = 39.9 vs 22.5% in fresh tissue). However, the PDP-dependent increase [(IV − II) − (III − II)] was obviously not influenced by the autolytic processes as it remained practically unchanged: 22.0 vs 18.6% in fresh tissue. Autolysis therefore increases the potential of the quaternary nitrogen compound HI-6 to reactivate AChE (most probably by increasing its membrane permeability) but, at least in our experimental conditions, PDP effects on HI-6 reactivation seem resistant to the autolytic processes. The reason for the poor HI-6 reactivation as well as lower PDP effects observed in human muscle (Table 2) must therefore result from certain species-specific differences and not from differences in autolytic damage.

Only in the endplate-free region of rat diaphragm was the PDP-dependent increase in HI-6 reactivation greater at high in comparison to low soman concentration (Table 2). A quantitatively much smaller increase was also found in human muscle. The observed soman concentration dependence suggests that under such conditions PDP acts by blocking soman depot, which is formed only at high soman concentration. In the endplate-rich part of the rat muscle preparations, where most of the AChE is bound to the extracellular basal lamina [6, 7], such a concentration dependence of the PDP effect could not be found suggesting that some mechanism of PDP action other than depot blockade predominates. It seems therefore that the location of AChE is also of some importance with regard to which mechanism of PDP action will predominate.

Surprisingly, AChE activity fell to 50.9 ± 13.3% (N = 4) of control activity when rat muscle preparations (endplate-free region) were treated for 20 min in PDP alone. The concentration of PDP during pretreatment was 18 μM in this experiment, but must have been considerably lower during AChE activity determination, since the preparations were washed in Ringer's after PDP pretreatment. The direct interactions of PDP with AChE suggested by these results were tested further in the experiments carried out on AChE from muscle homogenates and from the electric eel.

Histochemical demonstration of reactivated AChE activity in the preparations of the endplate-rich region of rat diaphragm

Figure 2 shows the intensity of AChE staining in the endplate region of muscle preparations treated as described before (see Fig. 1). As expected, the greatest staining intensity was in the untreated control preparation (Fig. 2 I). No AChE staining could be observed in the unreactivated soman-treated preparations (Fig. 2 II) after the same period

Table 2. HI-6 reactivation of AChE in whole muscle preparations (summarized from Table 1)

	Without PDP (III − II)		PDP (IV − II)		PDP-dependent increase in HI-6 reactivation [(IV − II) − (III − II)]	
Soman concn	High	Low	High	Low	High	Low
REPR	21.9	32.6	49.1	63.3	27.2	30.7
REPF	3.9	21.8	22.5	30.4	18.6	8.6
HM	13.8	11.8	24.5	18.4	10.7	6.6

Values are percentages calculated from the mean values in Table 1 according to the formulas in parentheses.

Columns are % AChE reactivation by HI-6 alone (without PDP), % AChE reactivation in the presence of PDP (PDP) and the corresponding % of the PDP effect (PDP-dependent increase in HI-6 reactivation).

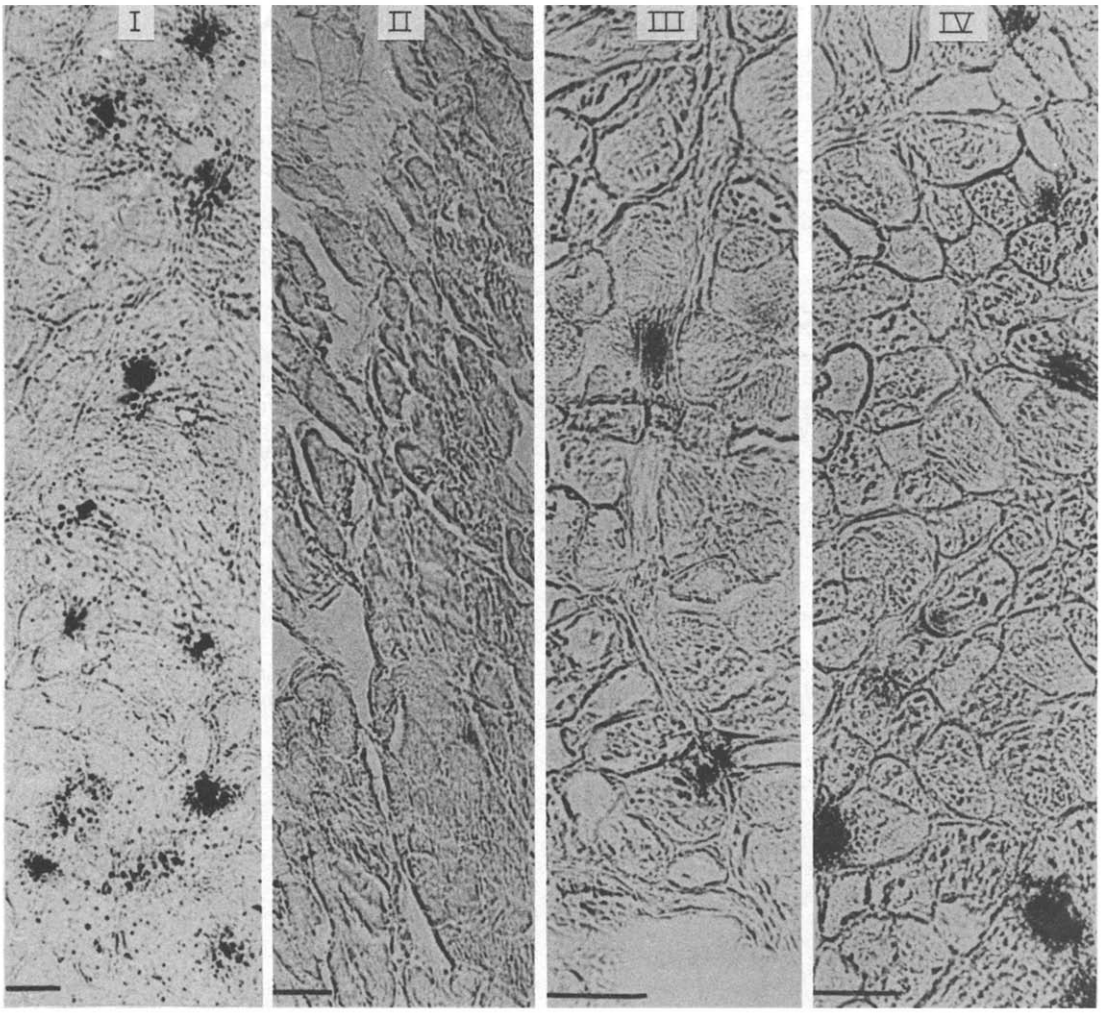

Fig. 2. Rat diaphragm: histochemical staining of AChE in the preparations isolated from the endplate-rich region. Histochemical precipitate demonstrating AChE activity (dark spots) is concentrated at the endplates. Roman numerals correspond to the individual experimental procedures, indicated by the same numerals in Fig. 1. (I) Control experiment; (II) preparations incubated in soman for 5 min; (III) preparations incubated for 30 min in HI-6 after 5 min incubation in soman; (IV) the same as in III except that prior to incubation in soman, muscle preparations were pretreated for 15 min in PDP at a concentration 10 times higher than that of soman. The time of incubation in the modified histochemical medium [15] was constant for all preparations: 15 min (bar = 100 μm).

of incubation in the histochemical medium. In HI-6-reactivated samples, the precipitate developed in the preparations pretreated with PDP prior to soman application (Fig. 2 IV) looked slightly more intense than that obtained after treatment with HI-6 alone (Fig. 2 III), which was in good accord with our biochemical determinations.

Homogenates of rat and human muscles, and purified AChE from the electric eel: effects of PDP on the AChE activity of the electric eel, and on the AChE activity, soman inhibition of AChE and reactivation by HI-6 in the homogenates

Seventy-four per cent of control AChE activity in rat and 45% of control AChE activity in human muscle homogenate became inhibited after 5 min incubation with 0.2 nM soman under our experimental conditions (Table 3 B). In the presence of PDP at a concentration 10 times higher than that of soman, soman inhibited only 50% of control activity in rat muscle homogenate (Table 3 C). Such a protective PDP effect was insignificant in the homogenate of human muscle under the same experimental conditions. HI-6 reactivation of soman-inhibited AChE, which was again more efficient in rat than in human muscle homogenate, was not improved in the presence of PDP in either human or rat muscle homogenate (Table 3 D and E). If 2 nM PDP was present in the incubation medium during incubation of homogenate with ACh, AChE

Table 3. Homogenates of rat diaphragm and of human intercostal muscles: the effects of PDP on the inhibition of AChE by soman and on the subsequent reactivation of AChE by HI-6

	A (%)	B (%)	C (%)	D (%)	E (%)
Rat diaphragm	100	25.9 ± 6.6	50.3 ± 12.1	87.2 ± 14.1	85.5 ± 15.2
Human intercostal muscles	100	55.5 ± 10.5	60.8 ± 17.4	64.5 ± 12.3	61.2 ± 13.8

Values in columns B–E are percentages of control (A). AChE activity (mean ± SD; N = 5 for rat muscle and N = 8 for human muscle) determined according to the procedures described in Materials and Methods. (B) % Control AChE activity after inhibition by soman; (C) inhibition by soman in the presence of PDP; (D) inhibition by soman in the presence of HI-6; (E) inhibition by soman in the presence of PDP and HI-6. All mixtures were incubated for 5 min at room temperature. Final concentrations of the reactants were as follows: soman 0.2 nM; PDP 2 nM; HI-6 0.5 mM.

In rat diaphragm muscle all mean values except A : D, A : E and D : E are significantly different from each other ($P < 0.05$). In human muscle the only significant differences were observed between A : B, A : C, A : D and A : E. All other mean values are not significantly different from each other ($P < 0.05$), using the same statistical tests as described in Table 1.

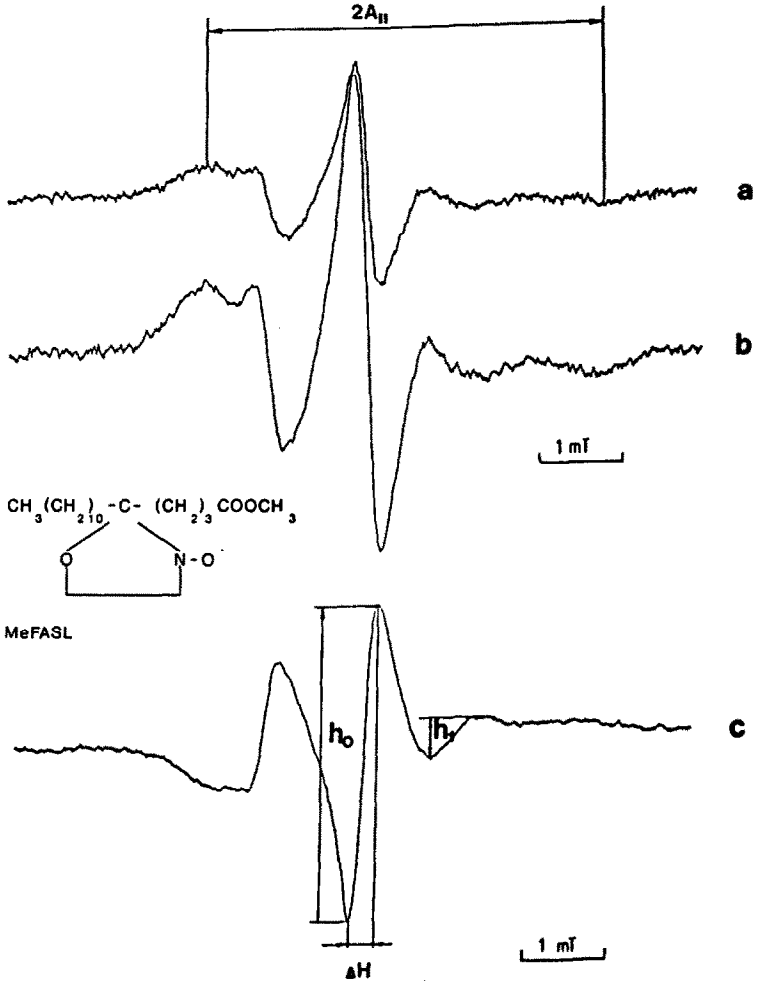

Fig. 3. Typical EPR spectra obtained in our experiments. MeFASL was incorporated into the membranes of human erythrocytes in the absence (a) and presence (b) of 100 μM PDP at 20° and in the absence of PDP at 31.5° (c). Spectra obtained in the presence of 10 μM soman and in the presence of a combination of 10 μM soman and 100 μM PDP were also not significantly different from control and are not known. Spectrometer settings: microwave power 10 mW, modulation frequency 100 kHz, modulation amplitude 1 mT; $2A_{//}$ indicates maximal hyperfine splitting. All relevant EPR parameters used in the present study are indicated.

activity fell to 33.7 ± 0.2% (N = 3) of control in rat and to 40.2% ± 1.9% (N = 3) in human muscle homogenate under our experimental conditions. On the other hand, no inhibitory effect of PDP on the hydrolysis of acetylthiocholine by the electric eel AChE could be observed under our experimental conditions.

EPR measurements

Information about putative interactions of PDP, soman and a combination of soman and PDP with the lipid bilayer of cell membranes was obtained from the measurements of $2A_{//}$ and τ_c, i.e. from the parameters characterizing membrane fluidity. A representative experiment is shown in Fig. 3. In the presence of 10 or 100 μM PDP an insignificant decrease in $2A_{//}$ ($2A_{//} < 0.04$ mT) was found for both human (20°) and rat (25°) erythrocytes. Furthermore, for human erythrocytes an insignificant increase in τ_c ($\Delta\tau_c < 0.1$ nsec) was found in the presence of PDP, while with soman alone at a concentration 10 times lower than that of PDP there was a tendency for τ_c to decrease. No effect on the parameters determined could be observed when both PDP and soman were applied together. In experiments with rat erythrocytes some changes in τ_c were observed in the presence of PDP alone, soman alone or both PDP and soman; however, these changes were not consistent with either increasing temperature or the substance tested; on average they were less than 0.1 nsec. From these results it could be concluded that under our experimental conditions no effect on the order and dynamics of phospholipid chains in membranes could be observed.

DISCUSSION

Pretreatment of muscle preparations with PDP increased the reactivation of soman-inhibited AChE by HI-6 in all our experiments. In the rat endplate-rich region biochemical determinations of AChE activities were confirmed by the histochemical technique. Our results suggest a more complex mechanism of the action of PDP than reported previously. A PDP-dependent increase in HI-6 reactivation, which was better expressed at high than at low soman concentration and could therefore be the result of blockade of soman depot, was observed in the endplate-free region of rat diaphragm and (less expressed) in human muscle under our conditions. The relative independence of these PDP effects from the concentration of soman found in the endplate-rich region preparations of rat diaphragm (Table 2) suggests an alternative mechanism of PDP action. Reduced inhibition of AChE activity in homogenates of rat diaphragm muscle obtained when PDP was present in the incubation medium during soman phosphorylation of AChE (Table 3) strongly suggests that PDP interferes with AChE phosphorylation by soman. The concentration of soman (0.2 nM) was so low under these conditions that most of it was consumed during 5 min incubation with homogenate and none of it was left to form a depot. Theoretically it is possible that structurally almost identical and 10 times more concentrated PDP (2 nM) non-covalently occupies soman binding site(s) on the AChE molecule. Such binding can interfere with AChE phosphorylation in various ways. The precise molecular mechanism of such interactions is an open question; however, a reported peripheral non-covalent organophosphate binding site on the AChE molecule, which (when occupied) reduces phosphorylation at the AChE active site [12], could be one candidate for the binding of PDP. It is relevant to mention here that a binding site other than the esteratic active center was demonstrated recently also for soman binding on the AChE molecule [19] suggesting complex interactions between AChE, soman and possibly PDP. Also, several characteristics of the described effects of PDP could be better explained by accepting the hypothesis that PDP, besides blocking soman depot, acts as an AChE binding ligand occupying specific binding sites. One such characteristic is the molecular structure specificity of soman simulators. It was reported that the more the structures of the simulators resembled that of soman the more they reduced its lethal effects; simulators without an intact pinacolyl group were inefficient [2, 3]. Only specific binding sites would demand such strict molecular specificity. Non-specific solubilization of soman into the "depot" site, which could be saturated and therefore blocked by PDP, would be less structure specific. At least one candidate for such unspecific soman depot, i.e. the lypophilic membrane compartment, can indeed be excluded after our EPR studies. On the other hand, when looking for the specific soman and PDP binding site in the muscle tissue one must recall that no other specific soman binding sites besides various serine esterases were ever reported in mammalian organisms, which again makes AChE a candidate for PDP binding. In one report, aliesterases were suggested as a possible site of soman depot [20] but non-covalent soman binding to these esterases was never directly demonstrated while covalent i.e. irreversible binding to their active sites is not consistent with the idea of depot. Another phenomenon speaking in favour of the hypothesis that PDP acts as a specific AChE binding ligand is the species specificity of the PDP effects. The poor PDP protection against soman in marmosets and guinea pigs in comparison to rats reported by other authors [5] is in accordance with our observations that, in comparison to rat, human muscle preparations as well as their homogenates show relative resistance to all effects of PDP tested (Tables 1–3). One should recall similar species specificity demonstrated for the AChE reaction with soman. It is known that AChE from rat and human muscle reacts with soman in different ways [21]; for example, "ageing", i.e. dealkylation of the pinacolyl group from the AChE–soman complex, is 10 times faster in human AChE than in that of rat [22]. It is difficult to accept the explanation that just by coincidence some other soman (and PDP) binding sites in the skeletal muscle besides AChE show exactly the same, i.e. rat versus human, species differences in interactions with soman and PDP.

Somewhat puzzling is the reduced AChE activity observed first on the muscle preparations from the

rat endplate-free region treated with 18 μM PDP and then also in homogenates of rat and human muscle when 2 nM PDP was present in the incubation medium during enzyme activity measurements. The observed AChE inhibition by 2 nM PDP in muscle homogenates, which was quantitatively comparable even with the inhibition by 0.2 nM soman (but under different experimental conditions: see Materials and Methods), suggests direct or indirect interactions of PDP with the AChE active center, but we were not able to confirm this assumption by the kinetic study on electric eel AChE, where no inhibition could be observed at much higher PDP concentrations (1.2 and 2.4 mM at the substrate concentration range of 40 μM–10 mM). Also, toxicity of PDP in rats is low [3], which could not be expected from the potential AChE inhibitor. On the other hand, increased mortality of rats was reported when they were given soman simulator 75 min after soman [1]. Authors explained this phenomenon by AChE inhibition by soman expelled with soman simulator from the soman depot, but inhibition by PDP of the residual AChE activity remaining after soman treatment would have the same effect. The observed discrepancy between PDP inhibition of AChE in the muscle preparations and crude muscle homogenates, and the absence of such inhibition of the electric eel AChE as observed in our experiments might have various explanations. It is well known that kinetic parameters determined on purified enzyme from electric organs differ significantly from the parameters determined on the *in situ* enzyme in mammalian tissues [23]. Our discrepancy could be explained by assuming that PDP inhibits AChE activity primarily through the peripheral non-covalent organophosphate binding site on the AChE molecule as mentioned above. It is important to note that AChE inhibitory effects of organophosphates which are mediated through this peripheral site could not be demonstrated on electric eel AChE but were found on the AChE in crude homogenate from mouse skeletal muscles [12]. This inhibition is probably too weak to cause serious toxic symptoms *in vivo* alone, but is high enough to potentiate soman toxicity, which explains the low toxicity of PDP when injected alone and its lethal effect when given after soman.

An extensive study will be necessary to prove our alternative explanation for the effects of PDP. It must be stressed that this explanation does not deny the existence of "classical" soman depot as formulated before [1, 4, 5]. Late mortality, inhibition of electric eel AChE in medium containing soman-intoxicated muscle and also our results on the PDP action in the endplate-free region of rat muscle preparation and (less so) in human muscle preparations could still be best explained by release of soman from some yet unidentified location that can be named soman depot. The reduced effect of PDP observed on human muscle speaks against the possible use of PDP as a prophylactic agent against soman intoxication in humans. However, if PDP works better in rats in comparison to humans only because PDP fits better to the peripheral center of rat AChE than to the equivalent peripheral center of the structurally different human AChE [24], a search for a soman simulator-like peripheral ligand that fits better than PDP to the peripheral center of human AChE would be justified.

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