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Phosphotriesterase, Pralidoxime-2-Chloride (2-PAM) and Eptastigmine Treatments and Their Combinations in DFP Intoxication

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The protective action of i.v. administered eptastigmine, an organophosphate hydrolase (phosphotriesterase), or pralidoxime-2-chloride (2-PAM) and their combination in acute diisopropylfluorophosphate (DFP) intoxication were evaluated in mice. The mice received the physostigmine derivative, eptastigmine (0.9 mg/kg body wt, i.v.), 10 min prior to the i.p. injection of DFP (1.8 mg/kg body wt). Phosphotriesterase (66 μmol/min $\times$ ml/g and 6 μg/g body wt) or 2-PAM (30 mg/kg body wt) were given i.v. 30 min after DFP. The animals also received atropine sc (37.5 mg/kg body wt) immediately after DFP. The cholinesterase (ChE) activities were not protected or reactivated by 2-PAM alone. The ChE activities in brain and plasma were protected by phosphotriesterase. Eptastigmine alone assisted the recovery of the brain ChE activities. Also the combination of eptastigmine–phosphotriesterase protected the brain enzymes. It did not, however, provide any additional protection compared with phosphotriesterase-treatment on its own. In brain, the combination of eptastigmine with 2-PAM resulted in partly restored enzyme activities 24 hr after DFP exposure. In plasma, eptastigmine did not prevent the inhibition of ChE by DFP. However, when it was combined with phosphotriesterase, it significantly promoted the recovery of plasma ChE activity. In lung and in erythrocytes, the various combinations of antidotes caused only minor changes in the ChE activities.

The acute toxicity of organophosphorus (OP) compounds in mammals is due to their irreversible inhibition of acetylcholinesterase (AChE, EC 3.1.1.7) in the nervous system, which leads to increased synaptic acetylcholine levels. The inhibition of AChE causes a cholinergic crisis producing several symptoms of poisoning, e.g., salivation, miosis, diarrhea, CNS depression, tremors, and respiratory failure, which may be lethal (Ecobichon, 1991).

Several different approaches have been tested in the therapy of OP poisoning. Traditional antidotes in OP poisonings include the combination of atropine and oximes like pralidoxime-2-chloride (2-PAM), obidoxime, and *bis*-pyridinium oxime (HI-6). The oximes dephosphorylate the OP-cholinesterase (ChE) complex. However, the ability of oximes to reactivate the OP-inhibited ChEs is not invariably satisfactory in all cases (Dunn and Sidell, 1989). Oximes fail to reactivate the inhibited ChE activity, if the OP-ChE complex has become aged. Moreover, large concentrations of 2-PAM may also inhibit ChEs (Matsubara and Horikoshi, 1984).

A potentially novel alternative OP antidote is to use exogenous enzymes such as purified AChE (Wolfe *et al.,* 1987, 1991, 1992; Ashani *et al.,* 1991a), butyrylcholinesterase (BChE; EC 3.1.1.8) (Ashani *et al.,* 1991a; Broomfield *et al.,* 1991; Raveh *et al.,* 1993), carboxylesterase (CaE; aliesterases, EC 3.1.1.1) (Maxwell *et al.,* 1987; Maxwell and Koplovitz, 1990; Maxwell, 1992) or OP hydrolases (aryldialkylphosphatase, EC 3.1.8.1, or arylester hydrolase, EC 3.1.1.2) (Ashani, 1992; Kaliste-Korhonen *et al.,* 1993; Tuovinen *et al.,* 1994, 1996a). Exogenous AChE, BChE, and CaE provide alternative binding sites for OPs and reduce the inhibition of endogenous body enzymes. While the abovementioned scavengers offer protection against the nerve agents, they have the disadvantage that they all have high molecular weights and react in a 1:1 ratio with the OPs. According to Broomfield (1992), about 500 mg of these enzymes must be active in the circulation for each milligram of OPs to be neutralized. Instead of simply acting as scavengers, OP hydrolases can actively hydrolyze OPs. This reduces the amount of OP in the body and prevents the inhibition of body esterases. Carbamates are also used in OP therapy. The centrally active carbamate, physostigmine, and the peripherally active pyridostigmine have been demonstrated to protect against the fatal effects of nerve agents such as soman (Leadbeater *et al.,* 1985).

In our previous studies, we have examined the ability of phosphotriesterase to protect mice against the toxicity of different OPs. Phosphotriesterase hydrolyzes several OPs, e.g., diisopropylfluorophosphate (DFP), paraoxon, sarin, so-

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man, and tabun. Our findings have shown that the phosphotriesterase-treated mice tolerated 33- to 50-fold higher paraoxon doses than controls (Tuovinen *et al.*, 1994). The i.v. injected phosphotriesterase protected the serum and brain ChE activities in mice, when it was administered before or after paraoxon (diethyl-*p*-nitrophenylphosphate) (Kaliste-Korhonen *et al.*, 1993; Tuovinen *et al.*, 1994, 1996a). It also prevented ChEs inhibition following DFP, sarin, or soman intoxications (Tuovinen *et al.*, 1994, 1996a). Moreover, we found that recovery of nonaged paraoxon inhibited ChEs was more rapid in phosphotriesterase-treated mice (Tuovinen *et al.*, 1994).

The aims of the present study were to investigate whether a combination of eptastigmine and phosphotriesterase or 2-PAM therapy could improve the ChE activities after DFP exposure. Theoretically, carbamate should increase the amount of OP in a free form, and phosphotriesterase should improve the possibility that the OP molecules become hydrolyzed before they inhibit the esterases. Moreover, we wanted to test the efficacy of eptastigmine–oxime combination and compare it to treatment with phosphotriesterase.

MATERIALS AND METHODS

Chemicals and materials. The derivative of physostigmine, heptylphysostigmine (eptastigmine; MF-201 tartrate; molecular weight 359), was kindly supplied by Mediolanum (Milano, Italy). The compound was dissolved in 0.9% NaCl (saline) solution. The phosphotriesterase enzyme is present in the soil bacteria, *Pseudomonas diminuta* and *Flavobacterium* sp. The phosphotriesterase coding gene from *P. diminuta* was expressed in *Escherichia coli* and a protein preparation was purified according to the method of Omburo *et al.* (1992) at Texas A&M University. Phosphotriesterase was dissolved in 50 mM Hepes buffer, pH 8.5, 1.0 mg/ml with 12,000 μmol/min $\times$ ml (12,000 μmol/min $\times$ mg prot.) of paraoxon hydrolyzing activity. In the purification system the naturally occurring Zn^{2+} was replaced with Co^{2+} (Omburo *et al.*, 1992). Pralidoxime-2-chloride (2-PAM) was purchased from the Aldrich-Chemical Co. (Steinheim, Germany) and dissolved in saline. DFP was purchased from Fluka AG (Buchs, Switzerland) and dissolved in olive oil. Acetylthiocholine, butyrylthiocholine, and propionylthiocholine iodide, the substrates for ChEs, and other reagents such as bovine albumin, atropine sulfate, 5,5-dithio-*bis*(2-nitrobenzoic acid) (DTNB), 4,4-dithiopyridine (PDS), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). Paraoxon, the substrate for paraoxonase and phosphotriesterase, was purchased from Ehrenstorfer (Augsburg, Germany). Astra 1397-HCl (10-diethylaminopropionyl phenothiazine-HCl), the specific inhibitor for BChE, was purchased from Astra (Södertälje, Sweden).

Animals. The animals used were 11- to 13-week-old CD_2F_1 male mice (25–29 g) (National Laboratory Animal Center, Kuopio, Finland). They were housed in groups of six animals in stainless-steel cages with aspen bedding (Tapvei Co., Kaavi, Finland). The mice were housed in a controlled environment: temperature 22 $\pm$ 1°C, relative humidity 50 $\pm$ 10%, and light period of 12 hr (07.00–19.00). Pellets of mouse diet (R36, Lactamin AB, Södertälje, Sweden) and water were freely available. This study was approved by the Ethical Committee for Animal Experiments of the University of Kuopio.

Experiments. Eptastigmine (0.9 mg/kg body wt in volume of approximately 0.2 ml saline) was injected into the tail vein 10 min prior to the i.p. injection of DFP (1.8 mg/kg body wt in volume of approximately 0.5 ml olive oil). Phosphotriesterase (66 μmol/min $\times$ ml/g body wt and 6 μg/g body wt in volume of approximately 0.15 ml) or 2-PAM (30 mg/kg body wt in a volume of approximately 0.15 ml) was injected i.v. 30 min after DFP. The control animals received i.v. saline and/or i.p. olive oil. To avoid possible toxic symptoms, the mice received sc atropine sulfate (37.5 mg/kg in a volume of approximately 0.15 ml saline) immediately after the OP injection. At 1, 3, 6, and 24 hr after the DFP exposure, the mice were sacrificed by carbon dioxide, and blood samples (approx. 1.0 ml) were drawn by cardiac puncture into heparinized tubes. The 25 μl of heparinized blood was diluted with 20 vol of 0.1% Triton in 50 mM sodium phosphate buffer. After that brain and lungs were quickly dissected and immediately frozen on dry ice. Plasma was separated with a hematocrit centrifuge at 1000*g* for 10 min at 4°C. The samples were stored at −75°C until measured.

Enzyme assays. ChE activities were measured spectrophotometrically at 410 nm at 37°C with the method of Ellman *et al.* (1961) using acetylthiocholine iodide as the substrate for brain and lung and butyrylthiocholine iodide for plasma. AChE activity in the blood was measured using propionylthiocholine iodide as the substrate according to the method of Augustinsson *et al.* (1978). AChE activity was measured after the inhibition of BChE with a specific inhibitor, Astra 1397-HCl. The blood samples were preincubated with the Astra-compound for 2 min before addition of the substrate. Paraoxon hydrolyzing activity was measured from plasma samples using paraoxon as the substrate. The assay method differed from the previous method (Tuovinen *et al.*, 1994). In this assay, the enzyme activity was measured in 0.0125 M borate buffer (pH 7.5) containing 50 μM cobalt in place of 300 μM calcium. The activity was spectrophotometrically monitored at 405 nm at 37°C.

Protein. Protein content of the tissue homogenates was measured by the dye-binding method of Bradford (1976).

Statistical analysis. The enzymatic data are presented as means $\pm$ SD. Comparison of enzyme activities between groups were calculated using one-way analysis of Kruskal Wallis and followed by Mann–Whitney U test. The *p*-value 0.05 or less was used as the level of statistical significance.

RESULTS

Phosphotriesterase (approx. 1800 μmol/min $\times$ ml/mouse) increased paraoxon hydrolyzing activity (POase) in mouse plasma by up to 10,000-fold compared to control level, measured half an hour after the administration of the enzyme. The activity rapidly decreased during the first 3 hr, and it was near to the control level at 24 hr (Fig. 1). Eptastigmine or 2-PAM had no effect on plasma POase activities (data not shown).

In brain, DFP inhibited AChE activity by 65% within the first hour, and the activity did not recover during the follow-up period (Fig. 2A). Eptastigmine (0.9 mg/kg) reduced brain AChE activity by 30% and the activity had recovered to 90% of control in 24 hr. Phosphotriesterase treatment increased the level of active AChE in brain by 20 to 40%. The activity had still not significantly recovered after 24 hr. The 2-PAM treatment did not offer any protection against DFP or reactivate brain AChE. Eptastigmine, even though it had an inhibitory effect on AChE, did not increase the inhibition of activity in DFP administered animals. After 24 hr, when the effect of eptastigmine had disappeared, the enzyme activity was increased from 45 to 75% in DFP intoxicated mice. The combination of eptastigmine–phospho-

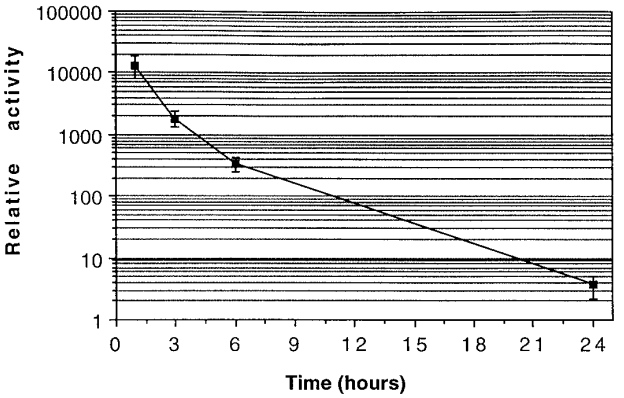

FIG. 1. The effect of phosphotriesterase (PTE; 66 μmol/min $\times$ ml/g body wt) administration on plasma paraoxon hydrolyzing activity (POase) in mice, when the relative activity in untreated mice was 1.0. PTE or saline were given i.v. 30 min after DFP or olive oil. Atropine sulfate (appr. 37.5 mg/kg) was injected sc immediately after DFP. POase activity in mice that had not received PTE was 165 $\pm$ 40 nmol/min $\times$ ml. Means $\pm$ SD are given, n = 5 or 6.

triesterase protected the enzyme activities in brain better than eptastigmine-2-PAM, but phosphotriesterase combined with eptastigmine did not, however, provide any additional protection when compared with phosphotriesterase- or eptastigmine-treatments on their own.

In lung, the ChE activity was maximally inhibited by DFP at the time point of 3 hr, and it showed virtually no sign of recovery (Fig. 2B). Eptastigmine reduced lung ChE activity by 50% during the first 80 min, and the activity recovered to the normal level within 24 hr. The antidotes and their different combinations did not significantly protect the ChE activity against DFP. At 24 hr after treatment, 2-PAM even increased the degree of inhibition. Phosphotriesterase, as well as eptastigmine, seemed, however, to have a slight protective effect on the activity, at the time point of 24 hr.

In erythrocytes, the maximum ChE inhibition after DFP injection occurred within 3 hr, after which the activity spontaneously recovered to the normal level during the next 3 hr (Fig. 2C). The inhibitory effect of eptastigmine was greater than that of DFP, and it also lasted for a longer time. Phosphotriesterase could not prevent the inhibition of enzyme caused by DFP exposure. 2-PAM–DFP-treated animals had generally the same activity levels as those treated with DFP alone. Surprisingly, however, 2-PAM seemed to slow down the recovery of enzyme activity. Eptastigmine–DFP treatment increased the inhibition of ChEs compared to mice receiving only DFP. The combination of eptastigmine with either phosphotriesterase or 2-PAM treatments did not significantly supplement the protective effects of the antidotes. For the first 6 hr the eptastigmine–2-PAM combination provided better protection of the enzyme activities than eptastigmine–phosphotriesterase.

In plasma, DFP inhibited BChE activity by 90% within the first hour, and the activity recovered spontaneously to 35% of the control between 6 and 24 hr (Fig. 2D). Eptastigmine inhibited plasma BChE activity by 60% during the first 80 min. However, by 6 hr the activity had recovered almost to normal. Phosphotriesterase protected the enzyme activities for the entire 24 hr. But this was not the case with 2-PAM. The eptastigmine treatment alone did not protect plasma ChE activities. It did, however, improve the protection efficacy of phosphotriesterase.

DISCUSSION

At the time point of 1 hr, the measured paraoxon hydrolyzing capacity of phosphotriesterase was greater than in our previous study (Tuovinen *et al.*, 1996a). This is due to the fact that $CoCl_2$ was substituted for $CaCl_2$ as cofactor in the assay method of POase. On the other hand, the control plasma POase activities were threefold greater in our previous study (496 vs. 165 nmol/min $\times$ ml). Presumably, Ca^{2+} is a better cofactor than Co^{2+} for the native POase (Gan *et al.*, 1990; Dave *et al.*, 1993). However, the difference in plasma POase activity after phosphotriesterase treatment may also be due to the differences in time schedules used. In the previous study, the plasma POase activities were measured 80 min after the phosphotriesterase administration, whereas now the activity was assayed 30 min after the phosphotriesterase treatment. The degradation of phosphotriesterase in plasma is known to take place very rapidly. According to Ashani *et al.* (1991b) the enzyme activity in plasma of mice which had received a phosphotriesterase-like enzyme stayed at a constant level only during the first 10–15 min after i.v. injection. The activity was cleared from the circulation within 150 min.

This study showed that phosphotriesterase alone decreased the acute toxicity of DFP better than 2-PAM. An i.v. injection of phosphotriesterase (both alone and in combination with eptastigmine) as late as 30 min after DFP exposure was able to protect brain ChE activity better than 2-PAM. Moreover, eptastigmine alone increased brain ChE activities which had been inhibited by DFP exposure. Harris *et al.* (1991) have reported that physostigmine can protect ChEs against tabun and sarin toxicity. Physostigmine has also been reported to decrease ChE inhibition in brain and serum in soman intoxication (Miller *et al.*, 1993). In our previous study (Tuovinen *et al.*, 1996b), eptastigmine–phosphotriesterase combination protected the ChE activities in plasma and lung, but not in brain, when phosphotriesterase was administered 10 min after DFP exposure. In the present study, phosphotriesterase was administered 30 min after DFP so that the enzyme activities would differ more from control level in phosphotriesterase-treated mice. When phosphotriesterase was now administered 20 min later, the ChEs were inhibited by 30% more in the DFP–phosphotriesterase

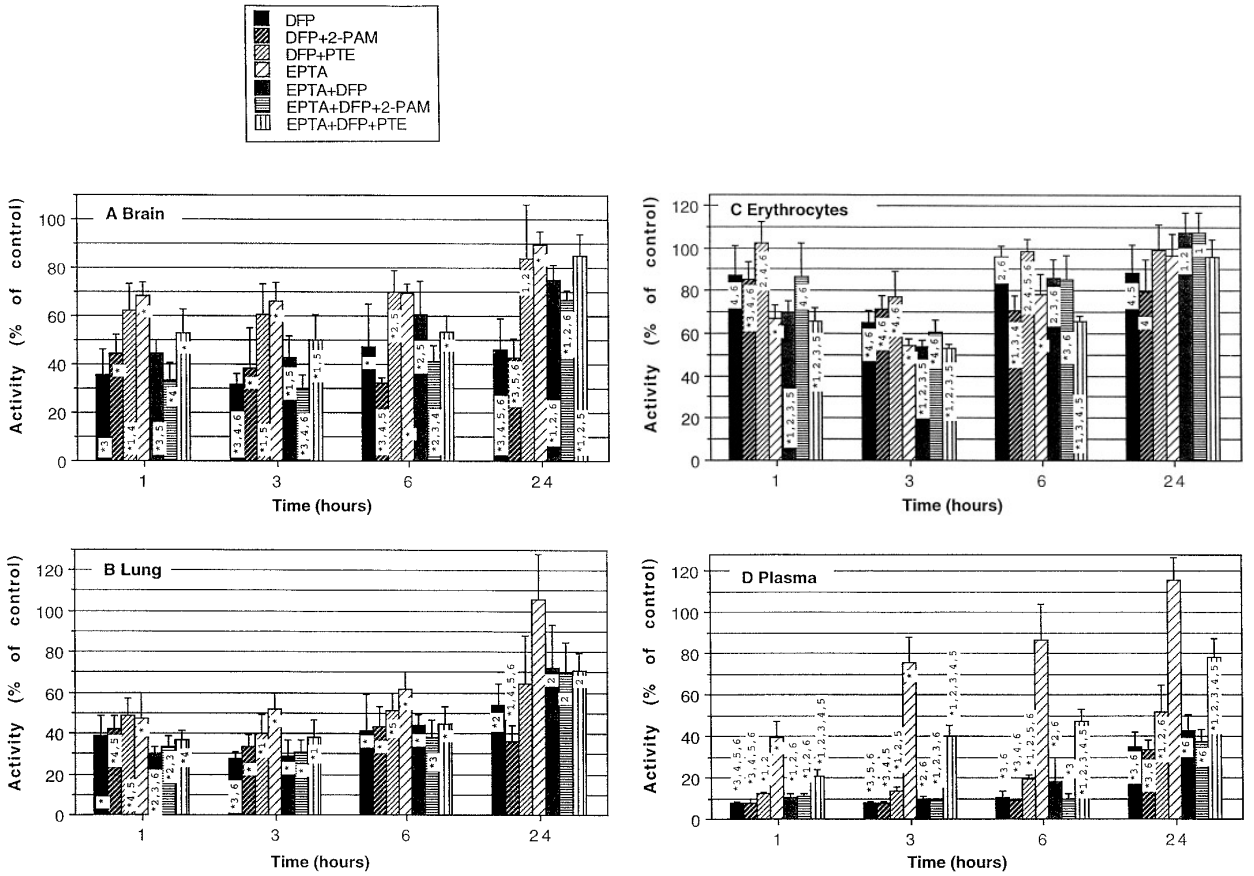

FIG. 2. The protective effect of heptylphysostigmine tartrate (EPTA; 0.9 mg/kg), PTE (66 μmol/min $\times$ ml/g body wt) and/or pralidoxime (2-PAM; 30 mg/kg) on brain AChE (A), lung AChE (B), erythrocyte AChE (C), and plasma BChE (D) activities after DFP (1.8 mg/kg) exposure in mice. EPTA was given i.v. 10 min prior to the ip injection of DFP. Thirty minutes later mice received PTE or 2-PAM. In addition, atropine sulfate (approx. 37.5 mg/kg) was injected sc immediately after DFP. Control activities were brain AChE 223 $\pm$ 11 nmol/min $\times$ mg protein, lung AChE 27 $\pm$ 4 nmol/min $\times$ mg protein, erythrocyte AChE 1 711 $\pm$ 268 nmol/min $\times$ ml, and plasma BChE 4 326 $\pm$ 318 nmol/min $\times$ ml. Means $\pm$ SD are given, $n = 5$ or 6. *$p < 0.05$ compared to the control; [1]$p < 0.05$ compared to the DFP-treated mice; [2]$p < 0.05$ compared to the DFP-2-PAM-treated mice; [3]$p < 0.05$ compared to the DFP-PTE-treated mice; [4]$p < 0.05$ compared to the EPTA-DFP-treated mice; [5]$p < 0.05$ compared to the EPTA-DFP-2-PAM-treated mice; [6]$p < 0.05$ compared to the EPTA-DFP-PTE-treated mice. EPTA-treated mice were compared only with the control mice.

group. Nonetheless, phosphotriesterase protected the enzyme activities. Eptastigmine combined with phosphotriesterase, however, prevented this additional enzyme inhibition in brain. Eptastigmine pretreatment would seem to provide more protection for ChE during the first critical minutes after DFP exposure.

In lung, all the therapies used seemed to be quite incapable of protecting the ChEs against DFP. After 24 hr, the eptastigmine-, eptastigmine–2-PAM-, and eptastigmine–phosphotriesterase-treated animals had higher enzyme activities. Presumably eptastigmine had prevented the irreversible inhibition of ChE by DFP. In our previous study the phosphotriesterase was given 10 min after DFP. This led to complete recovery of lung ChE activity in eptastigmine–phosphotriesterase-treated animals within 24 hr. Moreover, eptastigmine greatly improved the ability of phosphotriesterase to increase enzyme activity. In this study, phosphotriesterase was given 30 min after DFP. This provides further evidence that it is the first minutes after exposure which are critical for the effectiveness of phosphotriesterase in OP poisonings.

In erythrocytes, phosphotriesterase given alone showed the best antidotal effect against DFP.

In the present study, eptastigmine alone did not protect the enzyme activities in plasma against DFP inhibition. But, when eptastigmine was combined with phosphotriesterase, the combination protected and assisted the recovery of plasma ChE activity better than phosphotriesterase alone from the beginning of the experiment. By preventing the binding of DFP to ChEs, eptastigmine presumably led to an increase of the amount of free DFP in body fluids, and phosphotriesterase could thus hydrolyze this free OP more effectively.

The standard therapy against OP chemical warfare agent

poisoning in the U.S. army is a three-component regimen consisting of pyridostigmine, atropine, and 2-PAM. Surprisingly, 2-PAM did not reactivate the DFP-inhibited ChE activities. The various oximes have been reported to reactivate enzymes to a variable extent depending on which OP is involved. In general, the antidotal effect of HI-6 is based on its ability to reactivate peripheral ChEs, but it does not seem to have any great influence on the brain ChEs (Shih *et al.*, 1991; Sket, 1993; Sterling *et al.*, 1993). According to Dawson (1994), however, it was not important what kind of oxime was used when the treatment including pyridostigmine, atropine, and diazepam, was combined with obidoxime, HI-6, or 2-PAM. They all provided a 10-fold protection ratio in tabun, sarin, soman, and VX intoxications in guinea pigs.

Carbamates are reversible inhibitors of AChE (Brufani *et al.*, 1986). They compete with OPs for binding sites of ChE. They did not, however, additively potentiate the ChE inactivating effect of DFP. It may seem paradoxical to protect the ChE activities by using another inhibitor. This therapy has, however, proved to be effective in OP exposures, as shown also in this study with eptastigmine. The antidotal action of carbamates is attributed to their ability either to prevent irreversible phosphorylation of ChE by OPs (Green, 1983; Lennox *et al.*, 1985) or to temporarily block the ion channels associated with nicotinic cholinergic receptors or glutamatergic receptors in the neuromuscular junction (Karlsson *et al.*, 1984; Albuquerque *et al.*, 1985; Aracava *et al.*, 1987; Kawabuchi *et al.*, 1988).

The action of pyridostigmine is restricted to the peripheral nervous system. Some carbamates such as physostigmine, eptastigmine, and tacrine are able to penetrate the blood–brain barrier. In a similar manner, they may so protect also the brain ChE activities against OPs. Physostigmine is a well-known carbamate which has been tested also as an antidote for OPs. Eptastigmine was chosen for this study, since it is more lipophilic (rapid diffusion into the brain), less toxic, and has a longer duration of action than physostigmine in rodents (Marta *et al.*, 1988; Unni *et al.*, 1994; Sramek *et al.*, 1995). Moreover, our previous results showed that it caused only minimal changes in the behavioral activity of mice even though it augmented the inhibition of brain ChE. Overall, eptastigmine could represent a candidate carbamate compound for use in OP therapy. Hence, the decreased behavioral activity of mice was obviously not due to the ChE inhibition. One explanation for the behavioral effects of physostigmine could be its secondary actions on the cholinergic receptors.

In summary, an i.v. injection of phosphotriesterase as late as 30 min after DFP exposure was able to protect brain ChEs. Also eptastigmine alone assisted the recovery of brain ChE activities in mice with DFP intoxication. The combination of eptastigmine with phosphotriesterase did not improve the effect of phosphotriesterase except in plasma, where eptastigmine–phosphotriesterase greatly hastened the recovery of ChE activity. 2-PAM did not activate the ChEs which had been inhibited by DFP.

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