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Efficacy of Physostigmine as a Pretreatment for Organophosphate Poisoning

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MILLER, S. A., D. W. BLICK, S. Z. KERENYI AND M. R. MURPHY. *Efficacy of physostigmine as a pretreatment for organophosphate poisoning.* PHARMACOL BIOCHEM BEHAV 44(2) 343-347, 1993. – Continuous administration of the carbamate physostigmine, producing approximately 40% serum cholinesterase (ChE) inhibition, provides significant protection against the lethal effects of the organophosphorous nerve agent pinacolyl methylphosphonofluoridate (soman). Rats pretreated with physostigmine were also protected against the development of cholinergic symptoms and loss of body weight. Soman and physostigmine both inhibit ChE, yet animals pretreated with physostigmine exhibited less ChE inhibition in serum and brain than did animals exposed to soman alone. In addition, there did not appear to be any additive effect of presenting both anticholinesterases simultaneously. To further examine the effectiveness of physostigmine, we compared the results of this study with previously collected pyridostigmine data from our laboratory. This comparison indicates that physostigmine is more effective than pyridostigmine in protecting against the detrimental effects of soman.

Physostigmine Soman Lethality Organophosphate Pyridostigmine

THE carbamate, anticholinesterase (anti-ChE) physostigmine has been used to treat atropine overdose, poisoning with phenothiazines and tricyclic antidepressants (21), glaucoma (15), and myasthenia gravis (1), and recent studies indicate that it might be useful in the treatment of Alzheimer's disease (6, 7,23-25). This article concerns a newly proposed application of physostigmine, as a pretreatment against organophosphate (OP) poisoning to be used in conjunction with postexposure anticholinergic and oxime therapy (11).

Another carbamate, pyridostigmine bromide, has recently been fielded by the militaries of the United States and other countries as an orally administered protectant drug for possible OP nerve agent exposure (12). In combination with atropine and *N*-methyl-pyridinium-2-aldoxime chloride (2PAM), pyridostigmine is effective in protecting against lethality following acute OP exposure (8), but, probably because this compound does not cross the blood-brain barrier (BBB), it is ineffective protection against anti-ChE-induced performance deficits (3,14). The substitution of physostigmine for pyridostigmine might improve this situation.

Physostigmine, like pyridostigmine, reversibly inhibits ChE, protecting a critical percentage of the enzyme from irreversible binding to the OP, but unlike pyridostigmine physostigmine is a tertiary amine and as such readily crosses the BBB. Because of this property, it has been argued that physostigmine may afford protection to central as well as peripheral ChEs (3,10,14,16,26). However, ability to cross the BBB also raises the concern of possible unwanted centrally mediated psychological or physiological side effects.

Another possible complication with the use of one anti-ChE (e.g., pyridostigmine or physostigmine) to protect against the effects of another anti-ChE (e.g., OPs) is that at some point one might expect these two compounds to be additive in effect, in particular if the OP poisoning was repeated over a period of time. Of particular practical concern for military use of these drugs is whether prophylactic carbamates should continue to be administered during the possibility of repeated low-level exposure to warfare nerve agents. In previous articles, we addressed this question for pyridostigmine using both rat (14) and primate (3) models. Our results indicated that subjects exposed to continuous pyridostigmine and daily low-dose injections of the OP nerve agent pinacolyl methylphos-

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phonofluoridate (soman) did not differ from those exposed to soman alone. In the case of the rat, pyridostigmine did not offer protection against lethality, the development of convulsions, or weight loss, nor did it induce more severe effects (14). For the rhesus monkey, pyridostigmine did not offer appreciable protection against soman-induced performance decrements as indexed by performance on the Primate Equilibrium Platform (PEP) (3).

In this article, we report the results of an investigation of the efficacy of physostigmine as a pretreatment for soman exposure, as well as the possibility of an additive anti-ChE effect when it is used in conjunction with repeated low-dose soman. Further, our procedures were patterned after the study by Kerenyi et al. (14) so that we might compare the relative effectiveness of pyridostigmine and physostigmine.

METHOD

Subjects

Male Sprague-Dawley CD-VAF/Plus rats (300 ± 50 g) were obtained from Charles River (Wilmington, MA). They were housed three to a cage and maintained throughout the experiment on a standard laboratory diet and water ad lib. The animal quarters were climate controlled (22 ± 2°C) with a 12 L : 12 D cycle (light 0600–1800 h).

Materials

Physostigmine salicylate (Sigma Chemical Co., St. Louis, MO) was stored at 0–4°C and mixed as needed in a solution of 70% (1 : 2,000) glacial acetic acid in water, 20% propylene glycol, and 10% absolute ethyl alcohol. This mixture was then sonicated for 4 min to ensure that the physostigmine remained in solution. The just-mentioned solution, less the physostigmine, served as the control treatment.

Soman (>97% purity) was obtained at a concentration of 2 mg/ml from the United States Army Chemical Research Development and Engineering Center (USACR DEC, Aberdeen Proving Ground, MD). On the day of injection, further dilutions were made with 0.9% saline. Syringes were filled and packed in ice until injection (less than 1 h).

Procedures

Acute LD_{50} estimation. Six groups of animals (n = 6 each) received SC injections of soman in the nape of the neck for determination of an acute soman LD_{50} estimate. The doses were 60, 73.82, 90.81, 111.73, 137.45, and 169.1 μg/kg. The 24-h LD_{50} was calculated by the method of moving averages and interpolation (27).

Alzet pump validation: Serum and brain ChE determinations. In the experimental physostigmine group (PHY), each of 20 animals was implanted with a 0.2-ml, 7-day Alzet osmotic minipump filled with physostigmine for a steady dosing rate of 2.45 mg/kg/day. To avoid a bolus release of physostigmine, the osmotic minipumps were soaked in 37°C sterile distilled water for 5 min prior to implantation (13). Animals were anesthetized with ketamine, a small SC pocket was surgically prepared between the scapulae, and the pump was inserted with the delivery portal in a caudal orientation. The incision was closed with a wound clip. All surgery was performed under aseptic conditions. In a control vehicle-physostigmine group (VEH-PHY), each of 20 animals was implanted with a vehicle-filled pump in an identical manner.

Four animals from each group were decapitated 3, 4, 5, 6, and 7 days following implantation. Trunk blood was collected. Brains were removed and dissected on ice. The bilateral amygdalin, hippocampal, and pyriform cortical tissues were removed, homogenized in 1 ml 4°C 0.5% lubrol in sodium phosphate buffer, and frozen in a −70°C Forma freezer until the time of assay. The blood was centrifuged and serum samples were assayed for ChE activity using a modified Ellman et al. procedure (9). Because the physostigmine–ChE interaction is reversible, processing was done quickly (within 30 s of decapitation) to minimize the release of ChE. Also, all work was done on ice to slow the chemical reactions.

Brain ChE was assayed at a later date by a method similar to that used for the serum ChE assay (9). The homogenates were also assayed for protein content against a bovine serum albumin standard curve (17).

Five-day soman LD_{50} estimation. Rats were implanted with physostigmine- (2.45 mg/kg/day, which previously produced 40–50% serum ChE inhibition) or vehicle-filled osmotic minipumps. Soman injections began 3 days later, one injection per animal per day for 5 days. Soman doses ranged from 28 μg/kg/day to 102 μg/kg/day with three to eight animals per group. Animals were weighed before injection and observed for 1 h following injection. Symptoms of cholinergic toxicity were rated as: 1, fasciculations; 2, tremor; 3, lying prostrate; 4, convulsions; 5, death. The number of deaths were recorded at 1 and 24 h after each injection. One hour after the fifth soman injection, all surviving animals were decapitated for serum and brain ChE determination, as previously described.

RESULTS

Acute LD_{50} Estimation

The acute LD_{50} of soman was 132.78 μg/kg [95% confidence interval (CI): $118.29 \leq LD_{50} \leq 149.05$]. In the previous pyridostigmine study (14), the acute LD_{50} of soman was 121.5 μg/kg (95% CI: $97.2 \leq LD_{50} \leq 151.8$). Because each LD_{50} estimate falls within the 95% CIs for the other estimate, the acute soman LD_{50} estimates did not differ significantly.

Alzet Pump Validation: Serum and Brain ChE Determinations

As expected, the osmotic pumps (2.45 mg/kg/day) yielded a fairly constant level of ChE inhibition over a 7-day period (Fig. 1).

Five-Day Soman ± Physostigmine LD_{50} Determination

The LD_{50} estimates indicated a 5-day soman LD_{50} of 77.4 μg/kg (95% CI: $69.2 \leq LD_{50} \leq 90.3$) for the PHY group and 56.8 μg/kg (95% CI: $50.6 \leq LD_{50} \leq 64.8$) for the VEH–PHY group. Continuous administration of physostigmine via osmotic minipumps, therefore, provided a 1.36 protection ratio (PR) against five repeated soman exposures with the 95% CIs not overlapping, indicating a significant difference between the lethality of soman with and without the physostigmine pretreatment.

This finding is in sharp contrast to the results of our previous pyridostigmine study (14), where the 5-day LD_{50} of soman for the pyridostigmine group (PYR) and the vehicle-pyridostigmine group (VEH–PYR) were almost identical.

Because the acute soman LD_{50} estimates did not differ significantly between this study and our previous study with pyridostigmine (14), we further examined the data, hoping to find a means of comparing the two studies more directly. The only

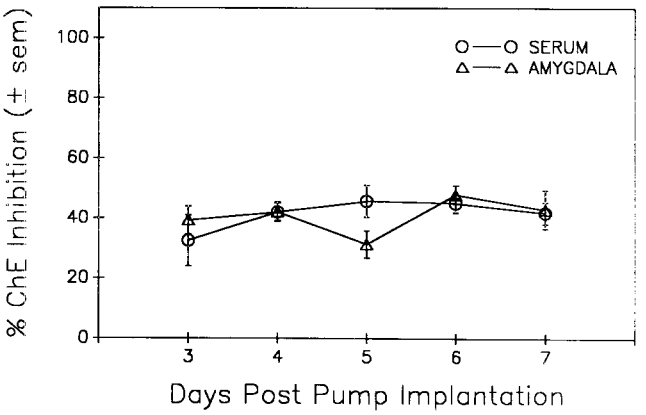

FIG. 1. Verification of the 7-day Alzet osmotic minipump: Cholinesterase (ChE) inhibition in the serum and amygdala as a result of continuous administration of physostigmine via Alzet osmotic minipumps. The pumps were determined to be functioning as expected because the ChE inhibition remained stable throughout the 7 days.

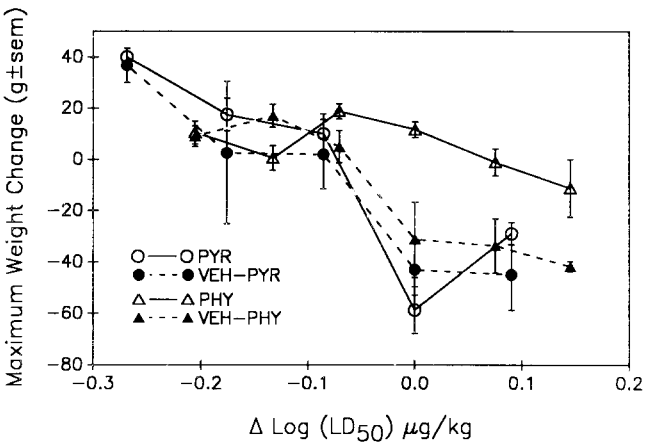

FIG. 2. Protection from weight loss: Pyridostigmine (PYR) vs. physostigmine (PHY). The critical difference between our previous study on pyridostigmine (14) and the current study on physostigmine is the difference between the LD_{50} estimates for the vehicle (VEH) groups in each case. Therefore, to directly compare the results of the two studies, soman doses were adjusted on the basis of the respective VEH LD_{50} estimates. The formula used was (log VEH LD_{50}) − (log soman dose) = Δlog LD_{50}. PYR data (PYR) and respective VEH data (VEH-PYR) are regraphed from our previous study (14).

obstacle was a difference in the 5-day soman LD_{50} estimates of the vehicles used in the two experiments. The group receiving the vehicle for pyridostigmine (14) had a lower 5-day soman LD_{50} (38.8 μg/kg; 95% CI: $34.5 \leq LD_{50} \leq 43.4$) than did the group receiving the vehicle for physostigmine (56.8 μg/kg; 95% CI: $50.6 \leq LD_{50} \leq 64.8$) in the current study. A possible explanation for this difference is that the vehicles themselves have some effect on the LD_{50}. In a preliminary portion of the physostigmine study, we compared the 5-day soman LD_{50} estimates for rats with vehicle-filled pumps and rats with no pumps. The LD_{50} estimates for these groups did not differ. Kerenyi et al. (14) did not have this comparison so it is possible that the vehicle used with pyridostigmine had a mild detrimental effect (i.e., lowered the LD_{50}). Another possible explanation for the difference in vehicle LD_{50}s is that the weights of animals differed in the two studies (physostigmine 300 ± 50 g vs. pyridostigmine 350 ± 50 g). Weight/age is known to have a rather substantial effect on the LD_{50} of soman. In any case, to better compare the data on pyridostigmine from the previous study with our data on physostigmine from the current study we transformed the data from both experiments on the basis of the 5-day soman LD_{50} estimates for their respective vehicles (VEH-PYR and VEH-PHY). The formula used was (log LD_{50}) − (log soman dose) = Δlog LD_{50}. All subsequent references (Figs. 2–5) to a comparison of data from the two studies is based upon this transformation.

Physostigmine protected against weight loss [General Linear Models (GLM); $p < 0.001$] (Fig. 2) and the development of cholinergic symptoms (χ^2; $p < 0.05$) for soman doses of 45, 53, and 63 μg/kg (Fig. 3) normally associated with OP intoxication, whereas pyridostigmine provided no protection from weight loss or symptom development.

A similar result is seen for serum ChE (Fig. 4). A GLM analysis indicated a significant soman dose effect ($p < 0.001$). It also showed that the PHY group had significantly less serum ChE inhibition than did the VEH-PHY group even though, in the PHY group, both the soman and physostigmine are inhibiting the enzyme ($p < 0.001$). As with weight loss and symptoms, physostigmine provided considerably more protection of serum ChE than did pyridostigmine (Fig. 4).

The analysis of brain ChE inhibition revealed a significant soman dose effect for all areas examined (GLM; $p < 0.044$, 0.001, and 0.008 for amygdala, hippocampus, and pyriform cortex, respectively), as well as a significant difference between the PHY and VEH-PHY groups for all areas studied ($p < 0.014$, 0.001, and 0.001 for the amygdala, hippocampus, and pyriform cortex, respectively). The soman dose-response curves are depicted in Fig. 5. Brain ChE data were not analyzed by Kerenyi et al. (14), so no comparisons are possible.

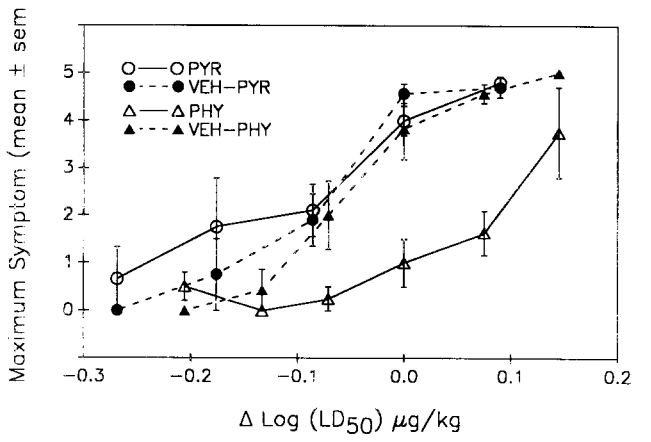

FIG. 3. Protection from cholinergic symptoms: Pyridostigmine (PYR) vs. physostigmine (PHY). Soman doses were adjusted on the basis of the respective vehicle (VEH) LD_{50} estimates. The formula used was (log VEH LD_{50}) − (log soman dose) = Δlog LD_{50}. Clearly, PHY pretreated animals had less severe symptomatology than either of the control groups or the PYR-pretreated group. PYR data (PYR) and respective VEH data (VEH-PYR) are regraphed from our previous study (14).

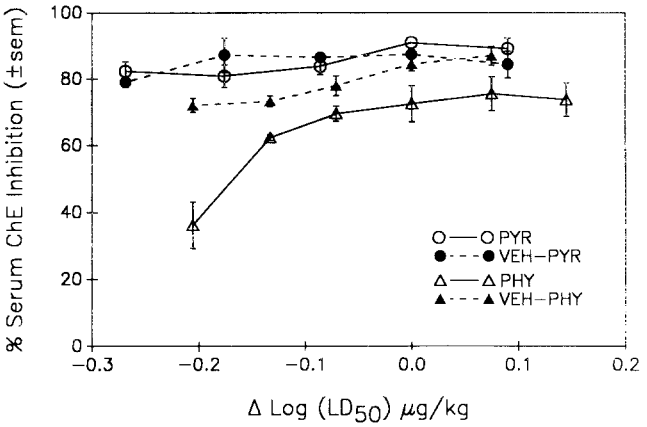

FIG. 4. Protection of serum cholinesterase (ChE): Pyridostigmine (PYR) vs. physostigmine (PHY). Soman doses were adjusted on the basis of the respective vehicle (VEH) LD_{50} estimates. The formula used was (log VEH LD_{50}) − (log soman dose) = Δlog LD_{50}. The PHY-pretreated group shows a protection from the effects of soman. PYR data (PYR) and respective VEH data (VEH–PYR) are regraphed from our previous study (14).

DISCUSSION

Although it might seem reasonable that simultaneous administration of two centrally acting anticholinesterases would likely lead to greater effects than either would if given alone, we found no evidence of additivity between physostigmine and soman. On the contrary, physostigmine seemed to diminish the effects of soman. Differences in the pharmacokinetics and pharmacodynamics of the two compounds may account for the lack of additivity.

Many investigators have found physostigmine to be superior to pyridostigmine in protecting against the effects of acute exposure to OPs (10,16). It has in general been accepted that this result is because physostigmine can penetrate the BBB and protect critical central stores of acetylcholinesterase (AChE). While the capability of crossing the BBB is a striking difference between the two compounds and most likely accounts for at least part of the results seen with physostigmine, there are a number of other fundamental differences between pyridostigmine and physostigmine. For example, the half-life for physostigmine plasma is 16–17 m (22), while the half-life of pyridostigmine is 1.9 h (2).

The pharmacokinetic and pharmacodynamic differences between pyridostigmine and physostigmine may also explain another somewhat surprising finding of this study, namely, the apparent protection of serum ChE by physostigmine, whereas in our previous study pyridostigmine offered no such protection (14). One might expect that because pyridostigmine and physostigmine are both carbamates inhibiting equivalent amounts of serum ChE prior to administration of soman there should be an equivalent increase in serum ChE inhibition for the two groups with the introduction of soman. The finding that the two carbamates differ so greatly in their interaction with soman and ChE in serum may be explainable by differences in their distribution, metabolism, and binding characteristics.

While a PR of 1.36 provided by physostigmine is modest protection against lethality, the ability of this carbamate to protect against weight loss and symptoms could be of particular importance. Miller et al. (19) and Murphy et al. (20) found

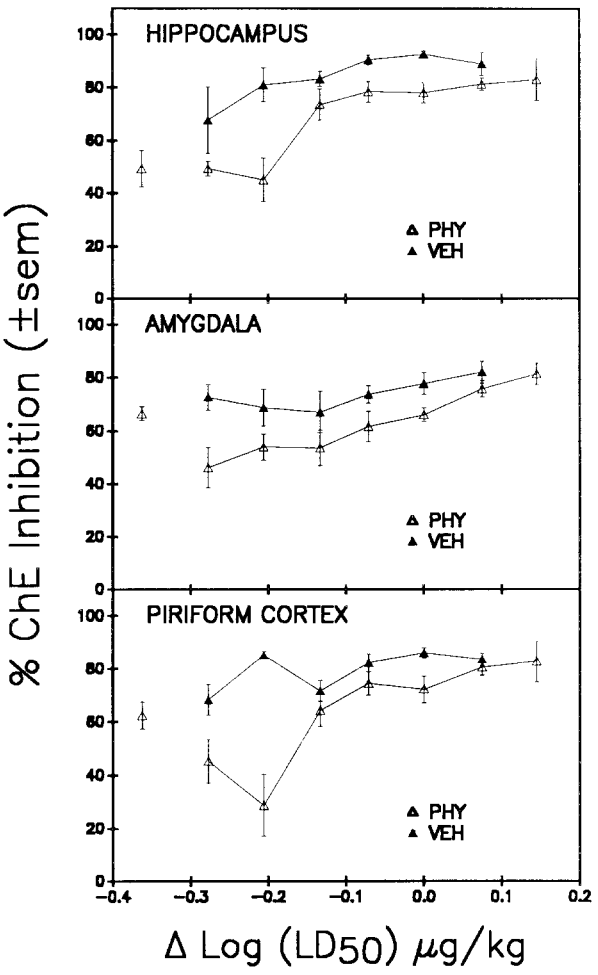

FIG. 5. Protection of brain cholinesterase (ChE). Soman doses were adjusted on the basis of the vehicle (VEH) LD_{50} estimate. The formula used was (log LD_{50}) − (log soman dose) = Δlog LD_{50}. In each brain area, physostigmine (PHY) pretreatment effectively reduced the inhibition of acetylcholinesterase by soman.

both weight loss and the appearance of convulsions to be correlated to the development of the devastating Soman Toxic Syndrome (STS). This syndrome is characterized by chronic spontaneous convulsions, hyperreactivity, a greatly increased sensitivity to stimulants such as caffeine and *d*-amphetamine, and extensive localized brain damage. Physostigmine's ability to protect against weight loss and symptoms (i.e., convulsions) could help prevent the development of STS.

Another advantage of physostigmine is its ability to maintain performance. Blick et al. (4) found modest yet significant protection of performance as measured by the PEP. Previous studies indicate that pyridostigmine protects against soman-induced lethality (14) but fails to provide operationally significant performance protection (3). Further, we found no physostigmine-induced deficits on the PEP at chronic physostigmine doses sufficient to inhibit serum ChE up to 40%.

Kadar et al. (11) proposed the use of physostigmine as prophylaxis against OP poisoning in the form of a transdermal patch. This method of administration eliminates the first-pass effect and would minimize the gastrointestinal side

effects reported by Desert Storm troops who took pyridostigmine tablets (12).

Even with the central actions of physostigmine, the protection is minimal and the addition of a reactivator (oxime) would probably be necessary. McDonough and Shih (18) suggest that scopolamine would provide additional protection.

Recent research suggests that, in the future, pretreatment with scavenger enzymes may be a better approach to OP protection than the use of any carbamate. We recently found (5) that rhesus monkeys pretreated with AChE purified from fetal bovine serum not only survived 27–32 μg/kg soman (four to five times the soman LD_{50}) but had no performance effects, visible cholinergic symptoms, or delayed effects. However, until ChE scavengers or other novel protectant drugs are practical, physostigmine would appear to offer significant advantages over pyridostigmine as a nerve agent pretreatment drug. In any case, we have shown with physostigmine, as had previously been shown for pyridostigmine, that no untoward or anti-ChE additive effects occur during repeated exposure to nerve agents in the presence of carbamate pretreatment.

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