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Side Effects of Physostigmine as a Pretreatment in Guinea Pigs

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PHILIPPENS, I. H. C. H. M., O. L. WOLTHUIS, R. W. BUSKER, J. P. LANGENBERG AND B. P. C. MELCHERS. *Side effects of physostigmine as a pretreatment in guinea pigs.* PHARMACOL BIOCHEM BEHAV **55**(1) 99–105, 1996.—To prevent incapacitation following nerve agent intoxications, it is proposed to replace pyridostigmine by the centrally active carbamate physostigmine (PHY). Behavioral and neurophysiological effects of PHY were determined and whether these effects would be counteracted by scopolamine. In addition, we compared them with the effects of another reversible cholinesterase (ChE) inhibitor ethyl-p-nitrophenylphosphoramidate (PNF). At similar levels of blood AChE inhibition, PHY caused a larger shuttlebox performance decrement than PNF, which was antagonized by scopolamine (0.1 mg/kg). SCO enhanced the PHY-induced increase of the auditory startle response, whereas PNF, with or without scopolamine, had no effect. In the EEG, PHY led to a power increase at the theta2-alpha1 band, also found after PNF, and at the theta1 band. SCO antagonized all EEG effects, but not the effects of PHY on visual evoked responses, in contrast to those of PNF. Based on the different effects of both inhibitors, it is suggested that at relevant doses several PHY-induced phenomena occur that are unrelated to AChE inhibition.

Physostigmine Behavior Neurophysiology Guinea pigs Cholinesterase

THE current pretreatment/treatment regime against intoxication with nerve agents, for instance, organophosphorus (OP) acetylcholinesterase (AChE) inhibitors, consists of a pretreatment with the carbamate pyridostigmine and treatment with atropine and an oxime. This treatment regime has been shown to be effective in a number of species (4,7–9). The suggested mechanism of action of pyridostigmine, a reversible AChE inhibitor, is to protect part of the AChE from binding with the irreversible OP AChE inhibitor. Due to its reversible binding with AChE by pyridostigmine, AChE activity may return fast enough to prevent lethality following an OP-intoxication. The drawback of pyridostigmine is that this drug poorly penetrates into the brain, due to its quartenary nitrogen atom. Therefore, pyridostigmine neither protects against the neurological symptoms nor against the severe behavioral incapacitation that usually follows OP intoxication.

Leadbeater et al. (13) investigated the possible use of physostigmine (PHY) as a pretreatment compound. This carbamate does penetrate into the central nervous system (CNS) and may protect AChE in the CNS from binding with an irreversible AChE inhibitor. A significant protection against lethality after sarin or soman intoxication was found. However, AChE inhibition in the CNS by the pretreatment may lead to unacceptable side effects. Leadbeater et al. (13) did find side effects in a swimming test, measuring gross motor performance. These effects of PHY could be counteracted by a low dose of scopolamine.

In the present study, behavioral and neurophysiological methods were used to determine the effects of physostigmine as well as those of ethyl-p-nitrophenyl phosphoramidate (PNF, see Fig. 1), a phosphoramidate that also reversibly inhibits AChE (12).

It was assumed that those effects that were similar for both compounds and/or could be counteracted by scopolamine would be due to AChE inhibition, whereas additional effects of these reversible inhibitors could be ascribed to other mechanisms of action of this compound. In this respect it should be noted that physostigmine has, in addition to its effects as a reversible AChE inhibitor, direct pharmacological effects, for example, on nicotinic ACh receptors (2,3,16).

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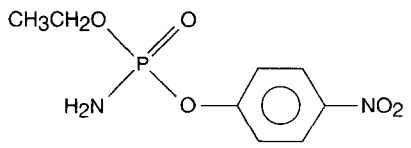

FIG. 1. Ethyl-p-nitrophenyl phosphoramidate (PNF).

METHOD

Animals

Male Dunkin-Hartley albino guinea pigs CrL:(HA)BR (Charles River) with a starting body weight of 350–400 g were used. The animals were kept one to a cage and the ambient temperature was regulated between 20–22°C. Relative humidity was monitored but not regulated and was always found to be higher than 50%.

Chemicals

Ethyl-p-nitrophenylphosphoramidate (PNF) was synthesized by Dr. H. P. Benschop PML TNO, Rijswijk (11). Eserine (Physostigmine) and scopolamine bromide were obtained from Sigma Chemical Co., St. Louis, MO.

Statistics

Analysis of variance (ANOVA) followed by a post hoc Newman–Keuls test was used for statistical comparisons. When the term significant is used, this means $p < 0.05$, two tailed.

General Procedure

In several series of experiments the following measurements were carried out: 1) shuttlebox performance, 2) startle responses, 3) electroencephalograms (EEG) and visual evoked responses (VER), and 4) AChE-activity of blood and brain (in parallel group of animals).

For methods, see below. All animals were tested before injection to obtain control values. Subsequently, on the basis of the results, three comparable groups of five to eight animals each were formed. Thereafter, the animals were subcutaneously injected with either saline, PHY, or PNF, and the AChE inhibitor in combination with scopolamine. Thirty minutes after injection the animals were tested in the shuttlebox task or their startle response was measured and thereafter, 45 min after injection, their EEG or VERs were recorded. All tests were repeated 24 h later.

Shuttlebox Performance

Shuttlebox performance was determined as described earlier (14). In short, an automated two-way shuttlebox was used, consisting of two equal compartments of 23 × 23 × 23 cm connected by a photo cell-guarded gate. The animals had to learn to avoid a stream of air (about 6 liter/s, air tube diameter 1 cm) aimed at their fur within 10 s after presentation of a tone. The animals were given 20 trials per day at an intertrial interval of 25 s (± 20% random). Criterion was 80% or more correct avoidance reactions (CARs).

Auditory Startle Response

The animals were exposed to 20 auditory startle pulses (120 dB, 10 Hz, 20 ms) while standing in a vertically mounted PVC-tube (diam. 7 cm, length 16.5 cm) and resting with their hindpaws on a platform. Startle responses of 100 ms duration were measured by registering the force exerted by the hind legs upon presentation of the stimulus. The measured parameters were ampl: the amplitude (force) of the response at its maximum, and AUC: area under the curve.

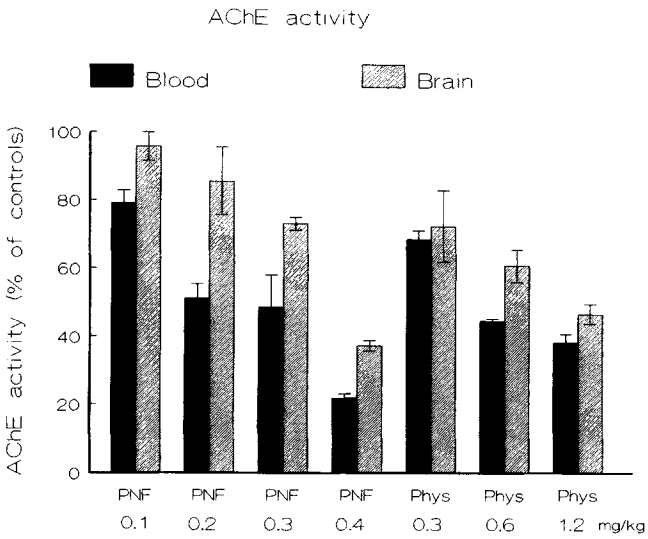

FIG. 2. The AChE activity in blood and brains of guinea pigs, 30 min after the SC injections of PNF (0.1, 0.2, 0.3, or 0.4 mg/kg) or physostigmine (Phys, 0.3, 0.6, or 1.2 mg/kg).

EEG Registrations and VER Measurements

Two days before the start of the experiments a small hole was drilled into the skull, 3 mm lateral to the sutura sagitalis and 8.5 mm caudal from the sutura frontoparietalis under halothane/N_2O anesthesia. The dura mater was left intact. A silver electrode was fixed into the hole with dental cement and a reference electrode—connected to earth—was fixed over the nasal cavity. The animals were immobilized in a vertically mounted PVC tube (as for the startle response). Fourier transformation (FFT), to obtain power spectra, was performed on line from five randomly chosen EEG epochs of 10 ms out of a total recording time of 5 min.

For the VER, the animals received 100 light stimuli of 1 Hz each. Following the stimuli the EEGs were registered during 250 ms and the responses were subsequently averaged.

EEG signals were amplified (50,000×), filtered (between 0.1–30 Hz for EEG and 0.1–300 Hz for VER) and fed into the ADC of an IBM-compatible PC; sampling frequency was 50 Hz for EEG and 1000 Hz for VER.

Determination of AChE Activity

Blood samples (5 μl) were obtained from the ear vein of the guinea pig, immediately mixed with 1% saponin (BDH, Poole, UK), frozen in liquid nitrogen and stored at −70°C. After appropiate dilution, AChE activity was assessed using a radiometric method (10). The ACh end concentration used was 12 μm;[^{3}H]ACh iodide (NEN, Dreieich, Germany) was diluted to a specific activity of 602 MBq·mmol^{-1}.

Ethopropazine (2.5 μM, St. Louis, MO) was used as a specific inhibitor of butyrylcholinesterase. Electric eel AChE was used as a reference.

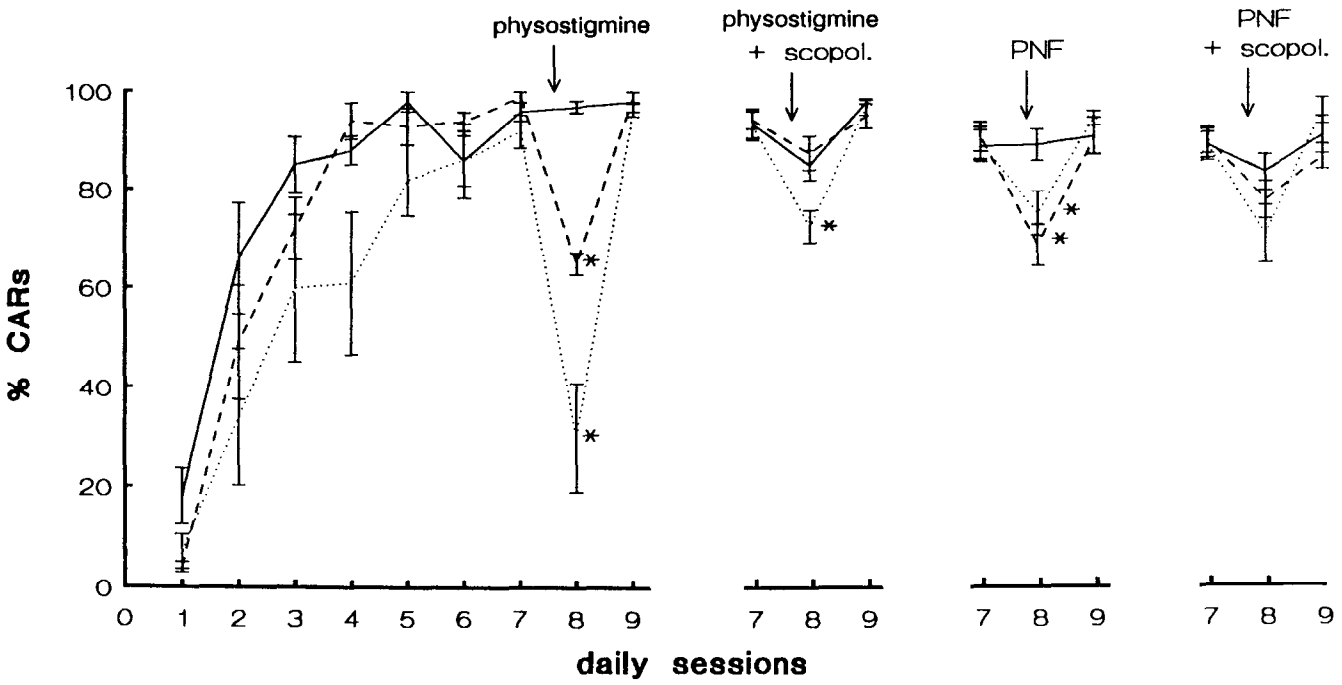

FIG. 3. Mean (± SEM) performance of guinea pigs in the shuttlebox task of four experiments. Animals were trained (CS, sound; UCS, air stream; 1 session/day, 20 trials/session) until > 80% CARs were reached, usually on day 7. On day 8, the drugs were injected and 30 min later another session started. Animals in Experiment 1 received saline (—), physostigmine 0.6 (- - -), or 1.2 mg/kg (·····) (n = 5/group). In Experiment 3, the animals received saline (—), PNF 0.2 (- - -), or 0.4 mg/kg (·····) (n = 8/group), and in Experiments 2 and 4 these drugs were combined with scopolamine 0.1 mg/kg (n = 8/group). All injections were subcutaneous. Arrow indicates the moment of injections. *Significantly different from the respective control groups using analysis of variance and Newman–Keuls post hoc test $p < 0.05$.

After decapitation the brain (cerebrum) was quickly isolated, weighed, and homogenized (1:10, w/v) in 50 mM Tris/HCl (Ph 7.4), 1 M NaCl, 5 mm EDTA, and 1% Triton X-100, using a Braun Melsungen Potter-Elvejhem type homogenizer (Melsungen, Germany). Homogenates were centrifuged for 10 min at 3000 × g and the supernatants were kept in liquid N_2 until determination of AChE activity was carried out as mentioned above.

RESULTS

The SC doses of the AChE inhibitors necessary to obtain therapeutically relevant levels of AChE inhibition in blood (13) were determined together with the associated levels of inhibition of brain AChE. It appeared that a SC dose of 0.6 mg/kg PHY led to the therapeutically desired level, for instance, about 40–50% inhibition of blood AChE. At this dose the brain AChE activity was reduced about 40% with respect to control levels (Fig. 2). A PNF dose of 0.2 mg/kg SC sufficed to reach 40–50% inhibition of blood AChE, whereas at that dose level AChE in the CNS was inhibited by only 15–20%. Because the level of AChE-inhibition in the brain following PNF 0.2 mg/kg was less than that following 0.6 mg/kg PHY, we also determined the AChE-inhibition in blood and brain after 0.3 and 0.4 mg/kg PNF. These doses of PNF caused a brain AChE inhibition of respectively 28 and 62%, which is more comparable with the brain AChE inhibition obtained with PHY 0.6 mg/kg.

In the shuttle-box task the acquisition rates for the four groups obtained in the experiments described in this article were very similar. After the performance criterium was reached, treatment with different doses of PHY led to a significantly dose-dependent performance decrease, $F(2, 12) = 26.63, p < 0.0001$. Newman–Keuls post hoc comparisons indicated a significant decrease after both dosages as compared to the control group, and a significant difference between these two treatment groups ($p < 0.05$) (Fig. 3).

PNF pretreatment, on the other hand, only showed a significant, $F(2, 20) = 6.54, p = 0.0065$, small performance decrement in the shuttlebox task, even at dose leading to a larger decrease of blood and brain AChE than caused by PHY (compare PHY 0.6 mg/kg and PNF 0.4 mg/kg, Fig. 3). The effects of PHY and PNF could be antagonized by a low, by itself sign-free (see Fig. 3), dose of scopolamine (0.1 mg/kg).

The effects of both PHY and PNF were reversible; 24 h after injection the performance was back to its preinjection value.

At a dose of 0.6 mg/kg, PHY caused a small increase of the startle response, which was increased dramatically when PHY was combined with scopolamine (0.1 mg/kg) [ampl.: $F(2, 21) = 12.41, p = 0.0003$ and AUC: $F(2, 21) = 23.8, p < 0.0001$] (Fig. 4). Newman–Keuls post hoc comparisons indicated a significant increase after PHY (0.6 mg/kg) + SCO (0.1 mg/kg) as compared to the control and the PHY (0.6 mg/kg) group ($p < 0.05$). All these effects were reversible; 24 h later the responses were on preinjection level. PNF, neither at a dose of 0.2 mg/kg, 0.3 mg/kg, nor 0.4 mg/kg (Fig. 4), had any effect on the startle response. The combination of scopolamine and PNF was also without effect [ampl.: $F(2, 15) = 0.63, p = 0.5466$ and AUC: $F(2, 15) = 0.06, p = 0.9374$]. Scopolamine in doses of 0.0 or 0.1 or 0.2 or 0.4 mg/kg had no effect, $F(3, 20) = 0.88$,

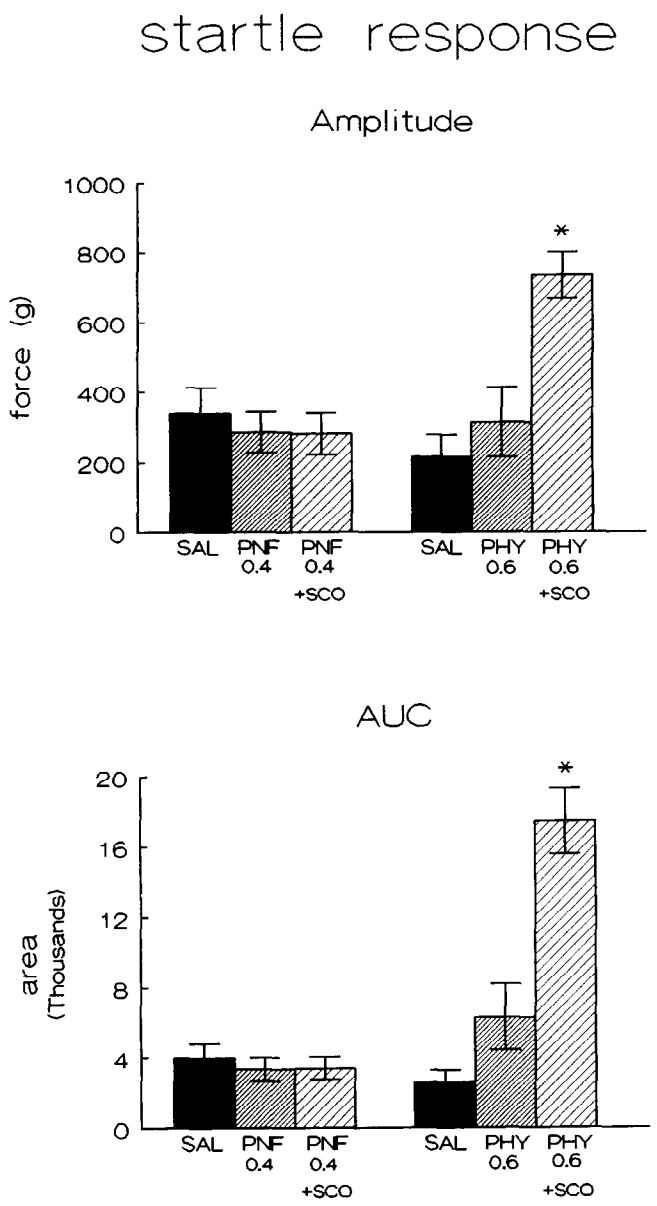

FIG. 4. Mean (± SEM) of the amplitude and AUC of the startle response (startle pulse: 120 db, 10 Hz, 20 ms). Registration of the effects 30 min after SC injection of: saline or PNF (0.4 mg/kg) or PNF (0.4 mg/kg) + SCO (0.1 mg/kg), $n = 8$ animals/group and saline or PHY (physostigmine) (0.6 mg/kg) or PHY (0.6 mg/kg) + SCO (0.1 mg/kg), $n = 8$ animals/group. *Significantly different using analysis of variance and Newman–Keuls post hoc test $p < 0.05$.

$p = 0.4668$] on the startle response amplitude (respectively, 48.3 ± 21.0, 80.7 ± 23.7, 99.9 ± 33.6, and 53.4 ± 22.2).

PHY, at a dose of 0.6 mg/kg, increased the EEG power in the frequency bands around 4 Hz and 7 Hz (Fig. 5A). Scopolamine (0.1 mg/kg) effectively antagonized those EEG effects: no significant differences were found between the EEG power spectra of the saline-treated and the PHY + scopolamine-treated group. PNF, in a dose of 0.4 mg/kg, led to an increase of the EEG power around 7 Hz (Fig. 5B). In contrast with PHY, no effect was seen on the power in the frequency band around 4 Hz. As was found for PHY, scopolamine antagonized those EEG effects. PNF in a lower dose of 0.2 or 0.3 mg/kg SC gave essentially the same effect. Twenty-four hours after the injections the EEG power spectra in both experiments were similar to their preinjection values.

Treatment with PHY (0.6 mg/kg) had various effects on the amplitude of the VERs. N1, N2, P2, and P3 peaks were slightly but not significantly reduced, whereas the amplitude of the N3 peak was significantly, $F(2, 21) = 14.91$, $p = 0.0001$, enhanced (PHY: −122 ± 15.6 μV; saline: −5 ± 19.2 μV). A combined treatment of PHY with scopolamine increased the effects on the amplitude, particularly on the N1 peak, instead of normalizing the VER (see Fig. 6A) [amplitude N1: PHY/SCO: −34 ± 8.6, saline: −80 ± 13.2, $F(2, 21) = 4.49$, $p = 0.0237$; N2: PHY/SCO: −18 ± 10.0, saline: 29 ± 12.2, $F(2, 21) = 4.71$, $p = 0.0204$; P3: PHY/SCO: 5 ± 7.4, saline: 54 ± 10.4, $F(2, 21) = 6.76$, $p = 0.0054$; N3: PHY/SCO: −97 ± 8.2, saline: −5 ± 19.2, $F(2, 21) = 14.91$, $p = 0.0001$]. The effects of PNF in a dose of 0.4 mg/kg (Fig. 6B) on the VER were different from those found after PHY. A delay in the latency was found, starting at the N3 peak (PNF: 123 ± 11.7 ms; saline: 89 ± 2.0 ms). Scopolamine antagonized this effect (97 ± 5.7 ms), $F(2, 14) = 4.80$, $p = 0.0258$.

DISCUSSION

In this study we used different neurophysiological and behavioral paradigms to test whether the reversible AChE inhibitor physostigmine (PHY) had central side effects. This carbamate penetrates the central nervous system (CNS) and may protect AChE in the CNS against binding with an irreversible AChE inhibitor. Because of the penetration in the CNS, there is a risk of unacceptable side effects of PHY. It was expected that the effects caused by the accumulation of ACh at muscarinic receptors would be counteracted by scopolamine.

The effects of this reversible AChE inhibitor were compared with those of another reversible AChE inhibitor with a completely different structure (PNF), at dose levels causing a similar level of AChE inhibition in blood or brain as at the relevant pretreatment dose of PHY used (0.6 mg/kg, SC).

It appeared that all effects of PNF treatment could be counteracted by scopolamine. In contrast with the results obtained after PNF, a number of effects of PHY were not counteracted by scopolamine. Notably, the large increases following the combination of PHY and scopolamine on the VER and particularly those on the startle responses were quite unexpected and are hard to explain. In this study, scopolamine did not have any effect on the startle response and, in general, effects of scopolamine on the startle response appear to be small, although slight increases as well as decreases have been reported (6). Muscarinic receptors appear not to be involved in the increased startle response. Because scopolamine is a competitive antagonist of ACh at the muscarinic receptor and because ACh accumulation also occurs at the nicotinic synapses, a complex picture emerges. Scopolamine may increase ACh release (5). That nicotinic receptors may be involved in the startle response may be inferred from the results of Acri et al. (1), who have shown that nicotine causes a dose-dependent increase of this response. However, any explanation should take into account that physostigmine, in addition to its effects as a reversible AChE inhibitor, has pharmacological effects unrelated to AChE inhibition. These effects may be due to blocking or stimulation of different receptors, for example, of nicotinic ACh receptors (2,3,16). Whatever the explanation, the different effects caused by PHY and PNF, whether

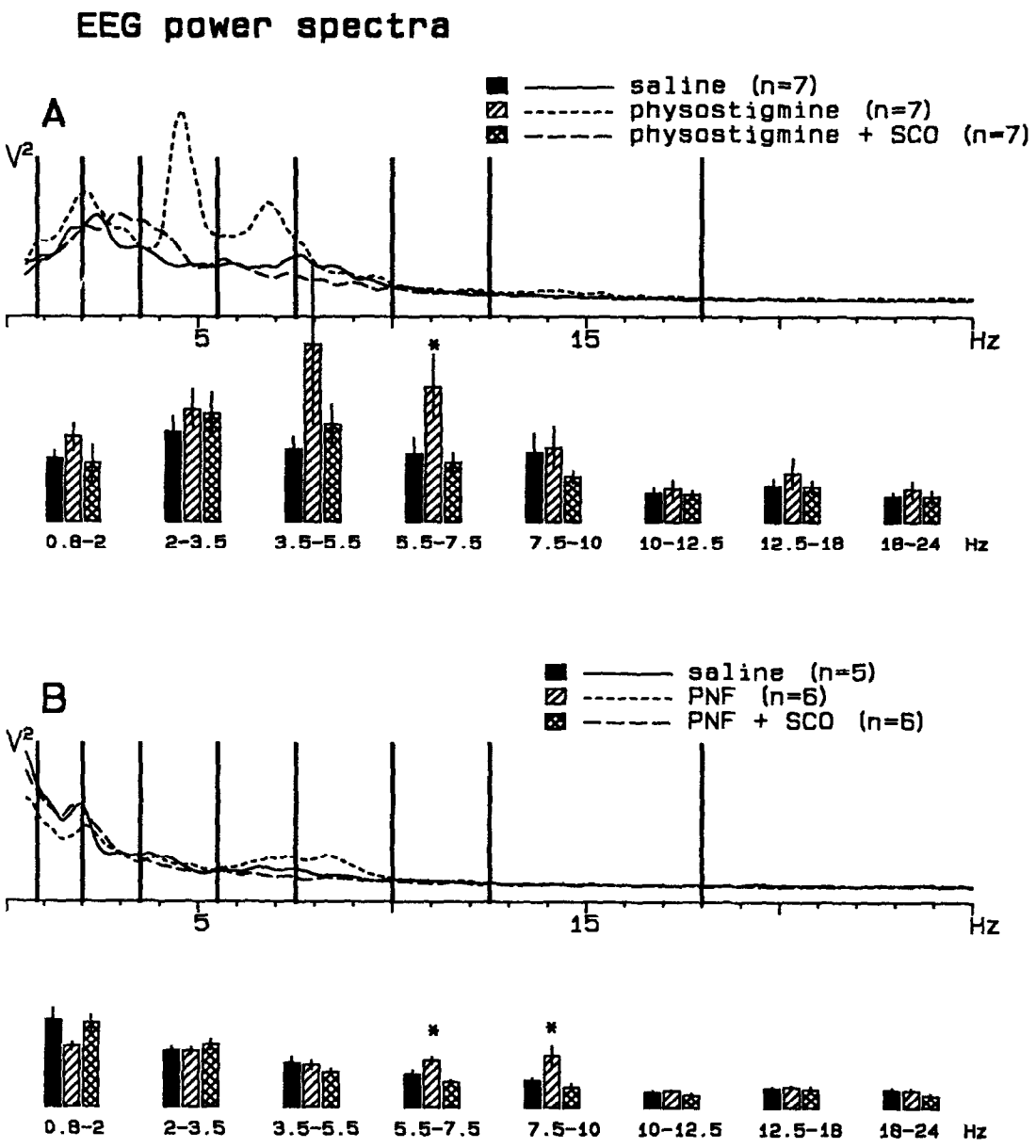

FIG. 5. Power spectra of EEGs obtained from guinea pigs. The vertical lines divide the frequency classes, the power of which is represented by bars at the bottom. Absolute power is shown (V^2). Registration of the effects 45 min after SC injection of (A) saline, physostigmine (0.6 mg/kg), or physostigmine (0.6 mg/kg) + SCO (scopolamine) (0.1 mg/kg) $n = 7$ animals/group; and (B) saline, PNF (0.4 mg/kg), or PNF (0.4 mg/kg) + SCO (0.1 mg/kg) $n = 6$ animals/group. *Significantly different from control and the combination with SCO using analysis of variance and Newman-Keuls post hoc test $p < 0.05$.

or not in combination with scopolamine, do indicate that more processes are involved than AChE-inhibition alone.

On shuttlebox performance, a SC injection of physostigmine causes a performance reduction. This reduction was appeared to be larger than the very small reduction found after administration of PNF at dose levels that caused approximately the same or even larger blood or brain AChE inhibition (Fig. 3). The finding that a sign-free dose of scopolamine could almost completely counteract the performance decrement caused by physostigmine, suggests that this decrement is the result of ACh accumulation at muscarinic receptors. After the combination of physostigmine plus scopolamine, a small effect remains that is approximately equal to the small effects of PNF.

On the EEG, physostigmine increases the EEG power in the frequency bands around 4 Hz and 7 Hz (Fig. 5), whereas PNF induces only a peak at 7 Hz. Because scopolamine effectively antagonized those EEG effects, both peaks at 4 and 7 Hz are likely to be due to ACh accumulation at the muscarinic sites. Activation of the central cholinergic system results in a shift in the power spectrum of the EEG from low to high frequencies (17). This is consistent with the results of our earlier studies, in which we tested a therapy against intoxica-

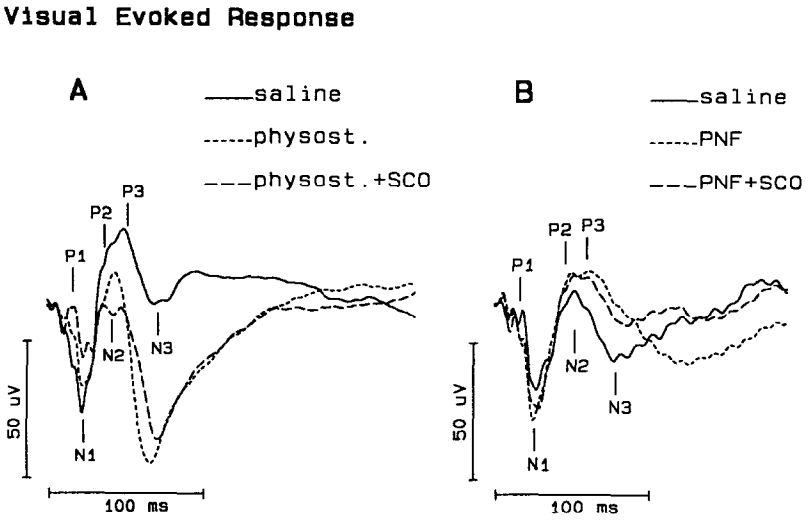

FIG. 6. The averaged visual evoked response (VER) curves from guinea pigs (n = 8 animals/group). Registration is shown 45 min after SC injection of (A) saline, physost. (physostigmine) (0.6 mg/kg), or physost. (0.6 mg/kg) + SCO (scopolamine) (0.1 mg/kg); and (B) saline, PNF (0.2 mg/kg), or PNF (0.2 mg/kg) + SCO (0.1 mg/kg). Significant effects using analysis of variance and Newman–Keuls post hoc test $p < 0.05$ were found for the amplitudes on the P3, N1, and N2 peak between saline and PHY + SCO, and on the peak N3 between saline and PHY or PHY + SCO. For the latency, a significant difference was found on the N3 peak between PNF and saline or PNF + SCO.

tion with an irreversible cholinesterase inhibitor. After a therapy with low doses of atropine and diazepam in intoxicated animals an increase of the delta power (1.5–3.4 Hz) was found that is indicative for neuropathology. After therapy with higher doses of these drugs, offering a better protection against convulsions and lethality, an increase of the alpha$_1$ power (7.5–9.9 Hz) was found that might reflect increased cortical cholinergic stimulation due to persisting cholinesterase inhibition (15). The simplest explanation for the occurrence of two peaks after physostigmine and only one peak after PNF may be that this is due to a difference of distribution in the CNS of these two compounds.

In conclusion, it will be clear that physostigmine causes several undesirable side effects that are not fully related to AChE inhibition; some, but not all, can be compensated by scopolamine. These effects occur at dose levels reported in the literature to be effective as a pretreatment against organophosphate intoxication.

If these results can be extrapolated to human it is expected that this will result in a high decree of jumpiness and increased startle reaction. The interpretation of the effects on the VER is uncertain, but might affect vision.

Despite the good profylactic efficacy of PHY, PHY appears to cause side effects that may make it less suited for use as a pretreatment.

However, recent experiments (to be published) show that upon subchronic administration these symptoms were not found.

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