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Subchronic Physostigmine Pretreatment in Guinea Pigs: Effective Against Soman and Without Side Effects

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PHILIPPENS, I. H. C. H. M., R. W. BUSKER, O. L. WOLTHUIS, B. OLIVIER, P. L. B. BRUIJNZEEL AND B. P. C. MELCHERS. *Subchronic physostigmine pretreatment in guinea pigs: Effective against soman and without side effects*. PHARMACOL BIOCHEM BEHAV **59**(4) 1061–1067, 1998.—The behavioral and neurophysiological effects of the subchronically administered cholinesterase-inhibitor physostigmine (PHY) (0.025 mg/kg/h) either with or without the muscarinergic antagonist scopolamine (SCO) (0.018 mg/kg/h) were determined in guinea pigs. In contrast to a single injection of PHY, subchronic application by osmotic minipumps of PHY, even without SCO, caused no behavioral or neurophysiological side effects. Also, the efficacy of such a pretreatment in counteracting soman-induced lethality and apparent symptoms of intoxication were determined. After subchronically administered PHY or PHY + SCO, the treated animals were protected against a 3 × LD_{50} dose of soman. © 1998 Elsevier Science Inc.

Behavior Cholinesterase Electrophysiology Guinea pig Muscarinergic receptor Physostigmine Pretreatment Scopolamine Soman Subchronic

EXPOSURE to nerve agents is currently not restricted to the battle field. When the world-wide chemical weapon convention is ratified by all the joining members the problem of the destruction of the chemical weapon depots will arise, which certainly will increase the risk of exposure. Because treatment for intoxications with at least some of these organophosphorus (OP) acetylcholinesterase (AChE) inhibitors is still far from ideal, research efforts are devoted towards finding an effective pretreatment. The mechanism of action of the currently available pretreatment pyridostigmine (PYR), a reversible AChE inhibitor, is to protect part of the AChE from binding with irreversible OP AChE inhibitors preventing phosphorylation of the enzyme. Due to the reversible binding of PYR with AChE, the activity of this enzyme may return fast enough to prevent lethality following OP intoxication (5,9,13,15). A serious drawback of PYR is that it poorly penetrates the brain due to its chemical structure (the presence of a quaternary nitrogen atom). Hence, PYR does not protect AChE in the central nervous system (CNS) from binding with an irreversible AChE inhibitor. For this reason physostigmine (PHY) has been suggested as an alternative (19). This compound possesses a tertiary nitrogen atom and does penetrate into the CNS. Furthermore, it has been shown to be effective against OP intoxication: a significant protection against lethality after sarin or soman intoxication was found (19). However, a pretreatment not only must be effective, but also be devoid of side effects, especially when given for a prolonged period. A single injection of a therapeutically relevant dose of PHY leads to unacceptable behavioral and neurophysiological side effects (25). Part of these undesirable effects appears to be caused by AChE inhibition in the CNS and are counteracted by scopolamine (SCO) (19,25). However, some effects of

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PHY appear unrelated to CNS AChE inhibition and are probably due to direct actions of this compound in contrast to the indirect AChE-inhibitory effects.

Because a bolus injection of PHY is not a very realistic pretreatment procedure, a more chronic application was considered and evaluated. In this study the presence or absence of PHY-induced side effects were determined in guinea pigs when administered subchronically with osmotic minipumps containing a therapeutically relevant dose of PHY, offering a blood AChE inhibition of approx. 40–50%, either with or without a low dose of SCO (also via the osmotic minipump). This level of blood AChE inhibition exceeds that of the recommended prophylactic level of AChE inhibition in case of PYR (10). However, a "worst" case approach seems to be appropriate when studying side effects of a pretreatment compound. Behavioral and neurophysiological test methods were used to determine the side effects of the various treatments. The efficacy of this pretreatment in counteracting soman-induced lethality and apparent symptoms of intoxication were also determined.

METHOD

Animals

Male Dunkin-Hartley albino guinea pigs CrL:(HA)BR (Charles River) with an initial body weight of 350–400 g were used. The animals were kept singly in a cage (Makrolon type IV). The ambient temperature was regulated between 20–22°C. Relative humidity was monitored but not regulated and was kept over 50%. Food and water were always available. An independent ethical committee approved the described experiments.

Drug Solutions and Implantation of Osmotic Minipumps

Physostigmine (eserine) and scopolamine bromide were obtained from Sigma, St. Louis, MO; Atropine Sulphate was obtained from ACF, Amsterdam, The Netherlands; Soman (O-pinacolyl methylphosphonofluoridate) was synthesized at the Prins Maurits Laboratory TNO (Dr. H. P. Benschop).

Alzet® Osmotic Minipumps with a constant delivery rate of 0.5 μl/h (Model 2002, Alza Corp., Palo Alto, CA) were used to deliver either the vehicle, PHY (0.025 mg/kg/h), or the combination of PHY (0.025 mg/kg/h) and SCO (0.018 mg/kg/h). The vehicle consisted of 20% propylene glycol, 10% ethanol, and 70% water (1 part glacial acetic acid in 2000 parts distilled water). The drugs used were solved in the vehicle. Because the animals gain weight during the 2 weeks of the experiments, the PHY and SCO concentrations were based on the estimated weight of the animals 1 week after implantation. This estimation was based on the normal growth curve for guinea pigs in our laboratory. The pumps were implanted subcutaneously under the skin on the backs of the animals under halothane/N_2O anesthesia. The wounds were sutured with woundclips.

General Procedure

The experiments were performed in five different treatment groups of animals as outlined in Table 1.

Electrodes for the measurement of EEG and visual evoked response (VER) were fitted 2 days before starting the training in the shuttlebox. To obtain control values, the EEG, VER, startle response, shuttlebox, and the blood AChE were registered/determined before implantation of the Alzet® minipumps. Subsequently, based on the obtained results, two matched subgroups of eight animals each were formed that showed no significant differences in any of the behavioral tests. Thereafter, Alzet® pumps, containing either vehicle or vehicle with PHY or a combination of PHY + SCO, were implanted. Two days after surgery daily testing in the shuttlebox task started. Registrations of EEG, VER, and startle response started at the same time and were repeated 5 and 10 days later.

TABLE 1

TEST PROTOCOL OF THE FIVE DIFFERENT TREATMENT GROUPS

Treatment	Tests	*n*
Subchronic PHY (0.025 mg/kg/h) + SCO (0.018 mg/kg/h)	EEG, VER, shuttlebox, blood AChE (t = day 13)	8
	Electrophysiology	5
	Startle, blood AChE (t = day 7), receptor binding	8
	Efficacy against soman, blood AChE (t = day 10)	8
	SCO plasma concentration	24
Subchronic PHY (0.025 mg/kg/h)	EEG, VER, shuttlebox, startle, receptor binding, and blood AChE (t = day 10)	8
	Efficacy against soman, blood AChE (t = day 10)	5
Subchronic SCO (0.018 mg/kg/h)	Receptor binding	8
Acute PHY (0.4 mg/kg) + SCO (0.1 mg/kg)	Efficacy against soman, blood AChE (t = day 10)	8
	SCO plasma concentration	8
Subchronic vehicle (control)	EEG, VER, shuttlebox, blood AChE (t = day 10)	8
	Electrophysiology	4
	Startle, blood AChE (t = day 7), receptor binding	8

For the electrophysiological measurements in the diaphragm muscle and for the muscarinic receptor binding experiments on brain tissue (cerebrum) (see below), the animals were sacrificed at the end of the experiment. The diaphragm muscle from the animals in which the shuttlebox, EEG, and VER were measured and the brain tissue from the animals in which the startle reflex was performed were used.

The efficacy of subchronic PHY + SCO pretreatment against soman-induced symptomatology and lethality was investigated 10 days after implantation of the osmotic minipumps ($n = 8$) and compared with that of acute PHY + SCO ($n = 8$) and subchronic PHY pretreatment ($n = 5$). The vehicle-treated animals received a subcutaneous injection with 0.4 mg/kg PHY and 0.1 mg/kg SCO (acute PHY + SCO group in Table 1), the subchronic PHY + SCO-pretreated animals received saline. Ten minutes later the osmotic pump was removed under halothane/N_2O anesthesia. Twenty minutes after removal of the pump blood samples were collected from the ear vein for the determination of blood AChE and the SCO plasma concentration. Subsequently, the animals were intoxicated with a subcutaneous (SC) injection of a 3 × LD_{50} dose of soman. The SC LD_{50} dose of soman in guinea pigs is 24.5 μg/kg as determined before (12). All animals received atropine therapy (17.4 mg/kg IM) 1 min after soman. The symptomatology was closely observed during the first 3 h by investigators unaware of the treatment and the lethality was determined at 24 and 48 h.

Shuttlebox Performance

An automated two-way shuttlebox, consisting of two equal compartments of 23 × 23 × 23 cm with rounded corners, connected by a photocell-guarded gate, was used. The animals had to learn how to avoid a stream of air (about 6 l/s, air tube diameter 1 cm) aimed at their fur within 10 s after presentation of a sound stimulus. The animals were given 20 trials per day at an intertrial interval of 20–30 s (random). The criterion was 80% or more correct avoidance reactions [for more details, see (24)]. Significant effects of drug treatment were expressed as % deviation of the control group.

Auditory Startle Response

The animals were exposed to 20 auditory startle pulses (120 dB, 10 kHz, 20 ms) while standing with their hindpaws on a platform in a vertically mounted PVC tube (diameter 7 cm, length 16.5 cm). The startle response of 100 ms duration was measured by a transducer connected with the platform, registering the force exerted by the hind legs upon presentation of the stimulus. The responses were digitized by the ADC of an IBM-compatible PC. For the evaluation of drug effects the area under the curve, the amplitude, and latencies of the startle response were compared with the values obtained in the control group.

EEG Registrations and Visual Evoked Response Measurements

Under halothane/N_2O anesthesia a silver electrode was fixed with dental cement into a small hole in the skull, 3 mm lateral to the sutura sagitalis and 8.5 mm caudal from the sutura frontoparietalis, leaving the dura mater intact. A reference electrode was fixed over the nasal cavity. The animals were immobilized in a vertically mounted PVC tube (as for the startle response). Fast Fourier transformation (FFT), to obtain power spectra, was performed on line from five randomly chosen EEG epochs of 10 s out of a total recording time of 5 min. The obtained power spectra of the guinea pigs were averaged per group and subdivided in frequency classes. For the evaluation of drug effects the power of each frequency class of the drug-treated group were compared with these of the control group.

For the visual evoked response (VER) the animals received 100 light stimuli at 1 Hz each. Following the stimuli the EEGs were registered during 250 ms and the responses were subsequently averaged. For the evaluation of drug effects the latencies and amplitudes of the positive (P1, P2, P3, P4) and negative (N1, N2, N3, N4) peaks of the drug-treated group and the control group were compared.

All 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 1 kHz for VER.

Electrophysiology

Electrophysiological recordings were made in the endplate zones of the diaphragms with conventional techniques, using microelectrodes filled with 3 M KCl having a resistance of 5–15 MΩ. The preparations were pinned down on Sylgard on the bottom of a small Petri dish containing Ringer solution (in mM: NaCl 116, KCl 3, $NaHCO_3$ 25, NaH_2PO_4 1, $MgSO_4$ 1, $CaCl_2$ 2, and glucose 11.1), at room temperature (18–22°C) and gassed constantly with 95% O_2–5% CO_2. To prevent contractions of the muscles, 2.3 μM μ conotoxine GIIIB (Sigma), which has a much higher affinity for the Na^+-channel of muscle than for that of the nerve (16), was added to the bath medium for at least 1 h. After washout of μ-conotoxine recordings were started. End-plate potentials (epps) were elicited by supramaximal stimulation of the phrenic nerve (50 μs, 0.5 Hz, 10 V), using a Grass S88 stimulator. Epps were sampled at 4 kHz and the miniature endplate potentials (mepps) at 16 kHz by means of an interface (CED-1401, Cambridge Electronic Design Ltd.) coupled to an IBM compatible personal computer. Commercially available software (Cambridge Electronic Design Ltd.) was used and the data were stored on diskette for later off-line analysis. The quantal content was calculated by the direct method, after correction of the epp amplitudes for nonlinear summation, assuming a reversal potential of −5 mV (22), by dividing the epp amplitude by the mepp amplitude. The latter was corrected for the occurrence of giant mepps, defined as mepps with an amplitude of more than twice the average mepp amplitude. Mepp frequency was assessed from stripchart recordings made on a Siemens inkjet recorder. The decay time constant of epps and the mepps was calculated from their decay phase, from 80 to 20% of the maximal amplitude, by making a least-squares fit of the natural logarithms of the data points in this region.

Receptor Binding Experiments

The cerebrum of the guinea pig was homogenized (1:20 w/v) in 30 mM HEPES buffer pH 7.4 containing 0.5 mM EGTA, centrifuged for 10 min at 1000 × g. Thereafter, the supernatant was centrifuged for 20 min at 48,000 × g. The resulting pellet was resuspended and the last centrifugation step repeated. Following resuspension, the protein concentration was adjusted to 2 mg/ml, and these membrane suspensions were kept at −80°C.

The number of muscarinic binding sites and the affinity of binding was determined for each individual animal by incubating membranes with 0.01–10 nM [^{3}H]-QNB. Nonspecific binding was determined in the presence of 10 mM atropine. The binding assays were performed in 20 mM HEPES. After incubation for 1 h at 25°C under continuous shaking, the incubation was terminated by rapid vacuum filtration over Whatman GF/C glass fiber filters using a Millipore (Etten-Leur, The Netherlands) sampling manifold. The filters were washed three times under vacuum with 3 ml of ice-cold buffer. The filters were placed in vials containing 5 ml scintillation cocktail, and counted at least 3 h later. Maximal binding, K_d, K_i, and pseudo-Hill coefficients were calculated after fitting the individual curves.

Determination of AChE Activity

Blood samples (25 μl) obtained from the ear vein of the guinea pig were immediately mixed with 1% saponin (BDH, Poole, UK), frozen in liquid nitrogen, and stored at −70°C. After appropriate dilutions, AChE activity was assessed using a radiometric method (18). The ACh end concentration used was 12 mM; [^{3}H]ACh iodide (NEN, Dreieich, Germany) was diluted to a specific activity of 602 MBq . $mmol^{-1}$. Electric eel AChE was used as reference.

Determination of Scopolamine Plasma Levels

Scopolamine plasma concentrations were determined using a radioreceptor assay (7). Guinea pig brain membranes were prepared as described earlier (32). Plasma (200 μl) obtained from ear vein blood samples were applied on C18 Sep-

pak columns (Waters). These were washed with 4 ml of water and eluted with 3 ml of methanol. The methanol was allowed to evaporate at 40°C, and the residues were dissolved in 500 μl of HEPES buffer. After dissolving, 150 μl was incubated with the brain membranes (1 mg/ml) in the presence of 255 pM [^{3}H]-QNB (Amersham; 49 Ci/mmol) in a total volume of 0.5 ml for 1 h at 25°C. Thereafter, the samples were treated as described in the receptor binding experiments. Scopolamine hydrobromide was used as a standard (1–600 nM in plasma), the recovery from plasma was 72 ± 15%.

Statistics

An analysis of variance (ANOVA) followed by a Newman–Keuls post hoc test was used to assess statistical significance in all the test systems used. For the symptomatology after soman intoxication a Fisher exact probability test was used. In both tests p values <0.05 were considered significant.

RESULTS

Blood AChE Inhibition After Physostigmine

The mean blood AChE inhibition of the subchronic PHY + SCO-treated animals was 38.9 ± 4.1% (n = 8), measured at day 7 after pump implantation, and 49.6 ± 2.5% (n = 8), measured at day 13 after pump implantation compared to the control values before pump implantation. The mean blood AChE inhibition of the PHY-treated animals, measured at day 10 after pump implantation, was 35.8 ± 4.9% (n = 8).

Scopolamine Plasma Levels

The plasma concentrations of SCO were determined after subchronic treatment using a minipump containing PHY (0.025 mg/kg/h)+ SCO (0.018 mg/kg/h). Plasma concentrations of SCO were also determined after single SC injections of PHY (0.4 mg/kg) + SCO (0.1 mg/kg) to correlate the subchronic SCO dose with the acute SCO dose from a previous study in which the side effects were also examined. Blood samples of the animals in which the efficacy of PHY pretreatment was investigated were used before the intoxication with soman. The scopolamine plasma concentration found in the guinea pigs after 10 days of subchronic PHY + SCO treatment was 45 ± 7 nM (n = 24); that in the acutely PHY + SCO-treated animals, 30 min after a SC injection with 0.1 mg/kg SCO, was 43 ± 9 nM (n = 8).

Effects on Behavioral and Neurophysiological Parameters

The measurements of possible behavioral and neurophysiological side effects were started 2 days after the implantation of the osmotic pump. The shuttlebox performance was tested every day. None of the daily sessions showed any aberration on the performance in the test groups treated with PHY (0.025 mg/kg/h) alone or PHY in combination with SCO (0.018 mg/kg/h) compared with the control group (an ANOVA analysis showed $p > 0.05$ in all the sessions). The startle response was measured 2, 5, and 10 days after pump insertion. Neither the amplitude of the response nor the latencies were affected in the test groups treated with PHY alone or the combination of PHY with SCO compared with the control group (at all test points an ANOVA analysis showed $p > 0.05$ for all the parameters). The neurophysiological tests were also performed 2, 5, and 10 days after pump insertion. The EEG activity was expressed as a power spectrum after FFT, which was subdivided in eight spectral bands. The total band power (V^2) in the different frequency classes of the test groups (PHY alone or the combination of PHY + SCO) showed no difference compared with the control groups. The visual evoked response (VER) consists of four positive and four negative peaks. The mean amplitude and the mean latency of each peak was measured and compared with the values obtained in the control group. Neither the amplitudes nor the latencies were affected in the test groups receiving PHY alone or the combination with SCO compared with the control group (at all registration points an ANOVA analysis showed $p > 0.05$ for all the different parameters in the EEG and VER).

Effects on Electrophysiological Parameters

Pretreatment with subchronic PHY + SCO, for a period of 13 days led to a significant decrease of the quantal content from 69.9 ± 4.3 in the preparations obtained from the control animals (n = 4), down to 51.5 ± 2.3 in the preparations of animals treated with subchronic PHY + SCO (n = 5). The mepp amplitude was not significantly different between the two groups (controls: 0.4 ± 0.04 mV; PHY + SCO: 0.5 ± 0.06 mV). The same held for the τ_{mepp} (controls: 2.2 ± 0.12 ms; PHY + SCO: 2.4 ± 0.13 ms) and the mepp frequency (controls: 0.40 ± 0.04 Hz; PHY + SCO: 0.50 ± 0.06 Hz).

Effects on Receptor Binding

The QNB binding experiments were performed on the brains of animals received a 14-day treatment with subchronic PHY or SCO or PHY + SCO. As shown in Table 2 , all these subchronic treatments resulted in a small (23–29%) but significant increase in the number of QNB binding sites compared to saline-treated controls, $F(3, 32) = 8.95$, $p = 0.0002$, Newman–Keuls posthoc analysis. An ANOVA analysis also showed a significant effect on the K_d, $F(3, 32) = 3.44$, $p = 0.028$. A Newman–Keuls post hoc analysis showed that the K_d in the PHY + SCO-treated group was significantly lower than the K_ds in the PHY- and SCO-treated groups. However, no significant difference with respect to the control group was found.

Efficacy Against a 3 × LD_{50} Dose of Soman

Pretreatment with acute PHY + SCO (vehicle in Alzet pumps) against 3 × LD_{50} soman. Most animals in this group only showed mild tremors, some ataxia, and muscle fasciculations, lasting from 10 min after intoxication till about 3 h after

TABLE 2

MUSCARINIC RECEPTOR BINDING AFTER A 14-DAY TREATMENT WITH PHY, SCO, OR PHY + SCO

	Muscarinic Receptor Binding		
Treatment	n	B_{max}	K_d
Control	8	100 ± 3	231 ± 14
PHY	8	125 ± 5*	314 ± 42
SCO	8	129 ± 7*	276 ± 33
PHY + SCO	8	123 ± 6*	131 ± 21†

The B_{max} is given as a percentage of control value; the K_d is given in pM.

* Significantly different from control value (Newman–Keuls).

† Significantly different from PHY and SCO treatment.

intoxication. Only one animal suffered from convulsions (lasting about 2 min) and dyspnoea. None of the animals died within 1 week after intoxication, and most animals were in a good condition. However, one animal did not recover fully. This animal appeared to suffer from a paresis of its front legs.

Pretreatment with subchronic PHY + SCO against 3 × LD_{50} soman. All animals showed mild to severe tremors, usually followed by a period of muscle fasciculations. Seven out of eight animals showed clear convulsive activity lasting for periods of 2 to about 20 min. Five animals suffered from a dyspnoea. Two animals, experiencing the most severe convulsive activity, died at 24.5 and between 29 and 38 h, respectively, after intoxication. The animals in this group showed significantly more frequently convulsive activities than those pretreated with acute PHY + SCO (Fisher exact probability test, $p < 0.05$, two tailed).

Pretreatment with subchronic PHY against 3 × LD_{50} soman. The animals of this group appeared to be in a better condition than the animals in the subchronic PHY + SCO treatment group. Two out of five animals showed convulsive activity lasting about 2 min, followed by a period of muscle fasciculatons and dyspnoea. One other animal showed slight tremors and some ataxia after a very short (≤1 min) period of mild convulsive activity. The remaining two animals only showed a slight ataxia for a period of 11–17 min, starting 3 and 9 min after intoxication, respectively. All animals were largely recovered about 30 min after intoxication and completely free of apparent symptoms 2 h after soman.

The AChE inhibition and effects on survival are summarized in Table 3. The AChE inhibition was significantly larger in the acutely pretreated group compared with the subchronically pretreated PHY + SCO group ($p < 0.05$, Newman–Keuls post hoc analysis). The results of earlier experiments with acute pyridostigmine (0.04 mg/kg SC) + SCO (0.1 mg/kg SC) are included in Table 3.

DISCUSSION

In this study the side effects as well as the efficacy against soman of subchronic pretreatment with physostigmine (PHY), given either alone or combined with scopolamine (SCO), were investigated.

No behavioral or neurophysiological side effects were found in the tests studied after subchronic pretreatment with PHY alone or the combination of PHY + SCO using a therapeutical dose that offers a good protection against OP intoxication. In the electrophysiological experiments on diaphragms from animals treated subchronically with the combination of PHY + SCO, a significant decrease of the quantal content was found compared with the control group. Subchronic treatment with PHY, SCO, or PHY + SCO also resulted in a significant increase in the number of muscarinic receptor (QNB) binding sites.

TABLE 3

PERCENT SURVIVAL AT 24 h AND 48 h AFTER A 3 × LD_{50} DOSE OF SOMAN IN ANIMALS PRETREATED WITH ACUTE PHY + SCO OR PYRIDOSTIGMINE (PYR) (0.04 mg/kg SC) + SCO, OR SUBCHRONIC PHY OR PHY + SCO

Pretreatment	*n*	AChE Inhibition	Survival After 3 × LD_{50} Soman	
			24 h	48 h
Acute PHY + SCO	8	50.5 ± 5.3%	100%	100%
Subchr PHY + SCO	8	33.3 ± 5.7%	100%	75%
Subchr PHY	5	35.8 ± 4.9%	100%	100%
Acute PYR + SCO	7	24.8 ± 4.0%	43%	43%

In an earlier study we already showed (25) that an acute dose of PHY, leading to similar levels of AChE inhibition as found in the present study, causes unacceptable behavioral and neurophysiological side effects. Only part of these effects were counteracted by SCO. Furthermore, an acute dose of ethyl-p-nitrophenyl phosphoramidate, another reversible AChE inhibitor, leading to similar levels of AChE inhibition in the brain, did not lead to similar effects as PHY treatment (25). This may indicate that some of the effects of PHY are unrelated to its AChE-inhibitory capacity.

In the present study, these side effects were not found after subchronic PHY or PHY + SCO pretreatment at levels of blood AChE inhibition that are in the range of or slightly higher than the recommended prophylactic levels. An explanation for the lack of these side effects may be found in the well-known phenomenon of tolerance against AChE inhibitors (8,34). Indeed, in our electrophysiological experiments on diaphragms obtained from subchronically treated animals with PHY + SCO, a clear decrease of the quantal content was found that is in accordance with earlier findings regarding tolerance to the effects of DFP (23), paraoxon (29), and also to the carbamate neostigmine (30). Interestingly, tolerance apparently was not only induced for those effects of PHY that could be ascribed to its AChE-inhibiting capacity but also for those effects, for example, the startle response, that were interpreted in an earlier study (25,26) to be unrelated to the levels of AChE-inhibition in the CNS.

In addition, an increase of the maximal binding of [^{3}H]-QNB was encountered after subchronic PHY, SCO, or PHY + SCO treatment, which is caused by an adaptation process (20). The increased number of muscarinic binding sites after SCO treatment appears to be in agreement with the findings of Baskin et al. (4). After (sub)chronic treatment with AChE inhibitors, usually a downregulation of muscarinic receptors is found (8,21,31). However, Bhat et al. (6) found no effect of subchronic PHY treatment, at a dose level leading to 62% inhibition of AChE, on [^{3}H]-QNB binding. The explanation of the upregulation might be found in the fact that the previous mentioned direct effect of PHY overruled the AChE-inhibiting effect. Indeed, the ED_50 of PHY's agonism at the nicotinic receptor appears to be lower than its IC_{50} of AChE inhibition (1). Furthermore, PHY has also a decreasing effect on the ACh release, which was found in the neuromuscular junction (27).

Despite the differences in side effects caused by either acutely or subchronically administered PHY, the protective effects of these pretreatment regimes against soman were very similar (Table 3). A small difference was found that may be explained by the differences between the levels of blood AChE inhibition in the different groups and the muscarinic receptor upregulation after subchronic pretreatment. These results are in full accordance with the results of others (14,21). Besides, PHY pretreatment is more effective than a pretreatment with PYR (Table 3). In the literature it has been reported that the protective ratio against soman intoxication of atropine sulphate alone is 1.5 compared with atropine sulphate + PYR, which is 5.2 (17).

In most of our experiments, PHY was combined with SCO. This was based on the results of Leadbeater (19) as well as on our own earlier study showing that SCO could counteract at

least some of the behavioral and neurophysiological side effects of PHY when given as one acute dose.

The dose of SCO we used (18 μg/kg/h) led to a plasma levels of 45 nM, which is much higher than those found by Wetherell (33), who treated guinea pigs with hyoscine at 6.5 μg/kg/h and found a SCO concentration of 2.3 nM. This may be explained by nonlinear kinetics of SCO. SCO is mainly excreted through metabolism into inactive metabolites (2). Saturation of hepatic elimination may cause an extraproportional increase of plasma SCO levels. Indeed, Wetherell (33) found a SCO concentration of 1.2 nM in animals treated with only 1.3 μg/kg/h.

From the present experiments it may be concluded that PHY offers a better protection against $3 \times LD_{50}$ soman compared with PYR pretreatment. The use of SCO as an adjunct pretreatment drug is not necessary regarding the side effects and efficacy against soman of the prophylactic regime. Furthermore, although after subchronic treatment of PHY an upregulation of muscarinic receptors was found, the behavioral performance and the neurophysiological activity were not affected compared to the acute pretreatment of PHY (25).

In conclusion, subchronic treatment with PHY seems to be a good alternative for the current pyridostigmine pretreatment. To increase the likelihood that these findings may be extrapolated to humans, it is imperative that they are substantiated by similar findings in other animal species closer related to humans. In this respect, the marmoset monkey seems to be the best alternative (3,28,35).

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