Physostigmine as a pretreatment against organophosphate-intoxication

A behavioural and neurophysiological study

Fysostigmine als voorbehandeling tegen organofosfaat intoxicatie

Een gedrags- en neurofysiologische studie

Ingrid Helena Christina Hubertina Maria Philippens

Promotoren: Prof. dr. B. Olivier (Universiteit Utrecht)
Prof. dr. R. A. A. Maes (Universiteit Utrecht)

Co-promotor: Dr. B. P. C. Melchers (PML-TNO)

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Physostigmine as a pretreatment against organophosphate-intoxication, TNO-PML
Thesis Utrecht University with a summary in Dutch
Subject headings: physostigmine / pretreatment / organophosphate / behaviour / neurophysiology

ISBN: 90-393-1669-4

Cover: "De beste bescherming is kennis" (photo of the corporate advertising campaign TNO, theme: defence research)
Printing: Drukkerij FEBO Druk (Enschede, Utrecht)

The research reported in this thesis was carried out at the department of pharmacology of the Medical Biological Laboratory TNO and the Prins Maurits Laboratory TNO Rijswijk and was supported by the Dutch Ministry of Defense. The views, opinions, and/or findings contained in this thesis are those of the authors and should not be considered to reflect the views of the Dutch Ministry of Defence.

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(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. dr. H.O. Voorma ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op vrijdag 11 december 1998 des middags te 12.45 uur.

door

Ingrid Helena Christina Hubertina Maria Philippens

Geboren op 3 juni 1964 te Heerlen

Beoordelingscommissie Dr. ir. H. P. Benschop
Prof. dr. A. R. Cools
Prof. dr. W. Seinen
Prof. dr. B. M. Spruijt
Prof. dr. J. L. Willems

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1

General introduction

Acetylcholine mediated neurotransmission

Chemical neurotransmission mediates communication between nerve cells and effector organs and is responsible for the function of muscles and more complex behaviour, such as learning and memory.

The ester acetylcholine (ACh) serves as a neurotransmitter in the central nervous system (CNS) and at peripheral junctions. It is synthesised in the region of the axonal terminals and is stored in concentrated ionic form in synaptic vesicles (De Robertis and Bennett, 1955). After stimulation of the axonal terminal (after a nerve impulse) the transmitter will be released (Fatt and Katz, 1952).

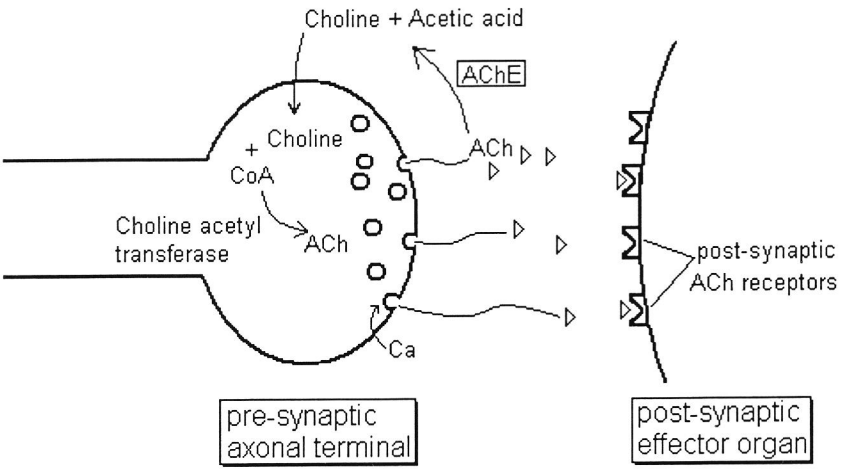


FIG. 1: Schematic representation of the cholinergic synapse illustrating the different steps in acetylcholine mediated neurotransmission.

Figure 1 shows the cholinergic synapse illustrating the different steps leading to ACh mediated neurotransmission. The release of ACh is initiated by the influx of calcium-ions in the intra-axonal medium. Calcium promotes fusion of the vesicular and axoplasmic membranes leading to exocytosis of ACh (Cooke *et al.*,1973). Released ACh diffuses across the synaptic or junctional cleft. At the postjunctional membrane it reacts with specialised receptor sites resulting in an increase of the ionic permeability, or conductance, of the membrane. Two main types of these ACh receptors exist, based on the mimicked effect of muscarine (from a toadstool) or nicotine (from tobacco): muscarinic and nicotinic receptors. These functionally and structurally distinct groups of ACh-receptors are further subdivided in a range of subtypes (see van den Beukel, 1998). To serve as a neurotransmitter, ACh must be removed or inactivated immediately after activation of the post-synaptic receptor. The enzyme acetylcholinesterase (AChE) is responsible for the hydrolysis of ACh to choline and acetic acid (see Fig. 2). The neurotransmitter ACh possesses a C=O that is necessary for attachment to the enzyme AChE. This binding takes place at the protein serine in the

catalytic centre of the esterase (Aldridge and Reiner, 1972). By interaction between the C atom of the carbonyl group and the serine hydroxyl group choline is split off, leaving the acetylated enzyme. This acetylated enzyme reacts rapidly with water to produce acetic acid and the regenerated active enzyme. Choline will be taken up by the pre-synaptic neuron from the extracellular fluid by active transport and can be used again for de novo synthesis of ACh. This final step in the synthesis of ACh (the acetylation of choline with acetyl coenzyme A) is catalysed by the enzyme choline acetyltransferase.

Newer insights, using X-ray analysis (Sussman *et al.*, 1991; Ripoll *et al.*, 1993) have revealed that the scheme in Fig. 2 not exactly illustrates the process of ACh-binding; the active site of AChE is situated in the centre of the enzyme at the bottom of a deep and narrow gorge. Actually, binding of the quaternary ammonium ion does not take place to a negatively charged "anionic" site, but rather to the "electron cloud" of the 14 aromatic residues that are situated in the gorge. Obviously, this newer insight of binding has also its consequences for the reactions outlined in Figures 3 and 4 (Soreq *et al.*, 1992). However, the three-dimensional structure of AChE, and changes in this structure during enzyme activation, are too complex and not directly relevant for this introduction.

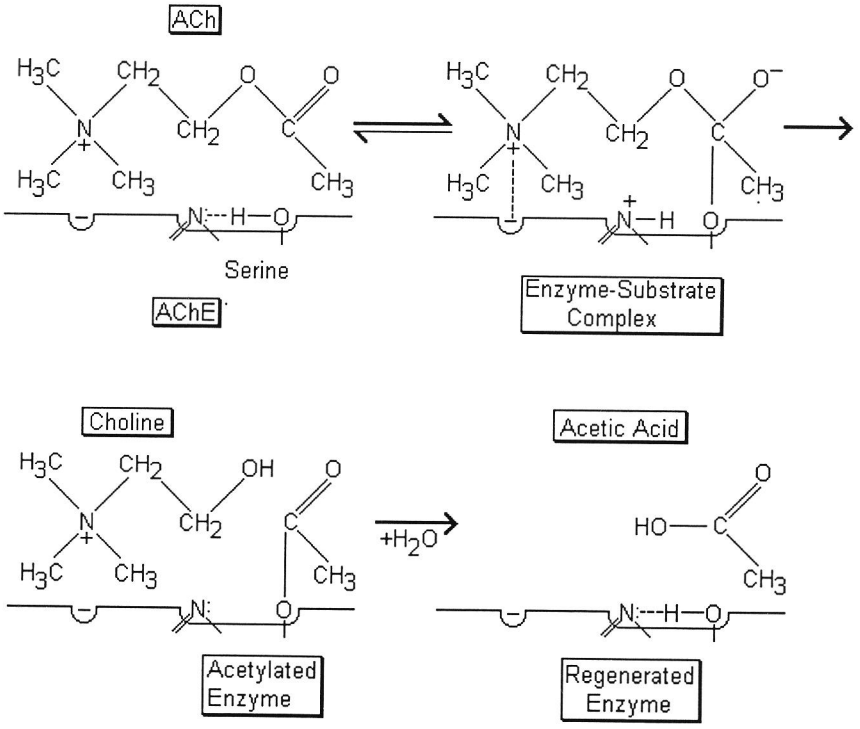


FIG. 2: Steps involved in the hydrolysis of ACh by AChE based upon previous kinetic, spectroscopic, and chemical modification studies.

Nerve gas intoxication

History of nerve gases

A casualty agent is a chemical compound that is used in military operations to incapacitate, seriously injure or kill personnel through its chemical properties. Nerve agents or organophosphates (OPs) belong to these casualty agents. The history of nerve agents started only 50 years ago. Shortly before World War II OPs were developed, first as agricultural insecticides and later, during the war, as potential chemical-warfare agents. They own their ancestry to the pesticide industry of Germany that developed these compounds for the German army. The first nerve gas was tabun (GA), followed by sarin (GB) and soman (GD). These agents are called the G-agents.

Tabun was one of the possible pesticides examined by dr G. Schrader, working at IG Farben Germany. After a small accident in the lab in 1937, dr Schrader and his assistants suffered from contracted pupils, dizziness, and acute dyspnoea. It took them three weeks to recover. After this accident they tested this drug on laboratory animals which all died within twenty minutes of exposure. The conclusion was that tabun was not suitable as a pesticide. This was the beginning of the synthesis and production of nerve agents. Tabun is a relatively slow acting nerve agent, but forms a stable cloud over a moderately persistent liquid. To achieve a fast acting nerve agent dr Schrader developed sarin. Sarin is extremely fast acting, and tends to evaporate at the same rate as water. The last of the three German nerve agents, soman, is a compromise between the first two agents: its evaporation rate is sufficient to allow it to remain in an area for about a day while it produces enough vapor mass. Fortunately, the German army never used these nerve gases in World War II. Later on, in 1952, the fourth agent VX (V-agent) was developed by the collaborative action of laboratories in the United Kingdom, the United States, and Canada. The most important characteristics of these four nerve agents are listed in Table I. In recent years other nerve agents, like cyclohexyl sarin (GF) or 2-dimethylaminoethyl-(dimethylamido)-phosphono fluoridate (GV) were synthesised. Due to the existence of these nerve agents and the likely development of new nerve agents, defence organisations are forced to search for adequate protection of their soldiers. Therefore, a lot of research has been carried out since World War II, on the external (protective gear / gasmask) and internal (prophylactic and therapeutic measures) protection.

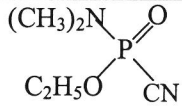
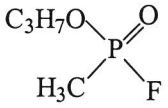
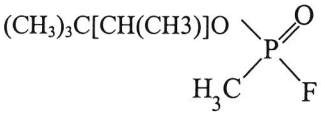
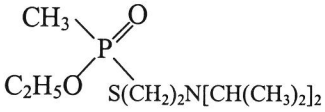
Risks of intoxication

Why still searching for a successful prophylaxis against nerve gas intoxication in a time in which a Chemical Weapons Convention is in preparation? Will there still be the risk of intoxication by a nerve gas? Unfortunately the answer has to be yes. Preparation of nerve gases is rather simple. Only small amounts are necessary to be lethal. Terrorist groups can easily use this kind of weapon when all basic substances are available. An example was the terrorist attack with the nerve gas sarin in the Tokyo underground in 1995. The sectarian group, responsible for this attack, had also used VX at another, earlier, occasion. The best cure is preventing the risk of exposure. In case of war this may be reached by the Chemical Weapons Convention that went into force in April 1997. However, in cases of war situations with countries that did not ratify the convention or terrorist actions there is no preventive measure. After ratification of the Chemical Weapons Convention the problem of destruction of these weapons will arise. Worldwide one may expect large nerve gas depots to be present,

particularly in the former Soviet Union and the United States. Destruction of these weapons can only be carried out when sufficient protective measures have been taken; one of them being a protective pretreatment against intoxication.

TABLE 1

Nerve agents: Comparative data

Common and chemical names	Structural formula	Volatility $mg.m^{-3}$ at 25°C	LCt_{50} $mg.min.m^{-3}$	ICt_{50} $mg.min.m^{-3}$
Tabun (GA) Ethyl N-dimethyl-phosphoramido cyanidate		610	400	300
Sarin (GB) Isopropyl methyl-phosphonofluoridate		22,000	100	75
Soman (GD) Pinacolyl methyl-phosphonofluoridate		3900	100	35
VX Ethyl S-2-diisopropyl aminoethyl methyl-phosphorothiolate		10.5	100	50

LCt50: median lethal concentration estimated for man

ICt50: median incapacitating concentration estimated for man

(data collected from Compton, 1987)

Symptoms of OP intoxication

The intoxication effects after OP exposure are mainly caused by the accumulation of ACh leading to the following symptoms (Taylor, 1996):

Peripheral muscarinergic: pupil narrowing (miosis), extensive salivation, bronchospasm, and intestinal spasm.

Peripheral nicotinergic: tremors, muscle fasciculations and tetanic muscle contractions leading to paralysis through the absence of neuromuscular transmission.

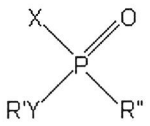
Central: extreme confusion, convulsions, unconsciousness, and/or paralysis of the respiratory centre that eventually can lead to death.

The early signs of OP intoxication are confusion, blurred vision due to miosis (pupil narrowing), and tightness of the chest. A severe OP intoxication leading to an AChE

inhibition of 95% or more will be lethal through respiratory failure, i.e. laryngospasm, bronchoconstriction, increased tracheobronchial and salivary secretion, and peripheral and central respiratory paralysis. A near lethal dose will cause irreversible brain damage in the cholinergic areas such as the hippocampus. This can lead to concentration, memory, and learning disabilities. Another effect of these anti-AChE agents can be a cholinomimetic action of the muscarinic type at autonomic effector organs.

Mechanism of action of OPs

Nerve agents are derivatives of phosphoric acid, all with the general formula (Schrader, 1952):



Y = O, S
X = F, CN, N_3, $S(CH_2)_2S^+R_2$", $S(CH_2)_nNR_2$"
R' = alkyl, cycloalkyl or H
R"= alkyl, dialkylamino

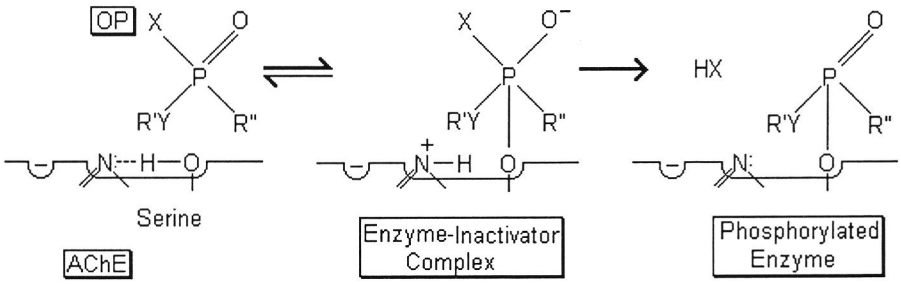


FIG. 3: Steps involved in the inhibition of AChE by OP-agents based upon previous kinetic, spectroscopic, and chemical modification studies.

The nerve agents are alkyl esters of either a dialkylaminocyanophosphoric acid (tabun), alkylfluorophosphoric acids (sarin and soman), or a S-dialkylaminoethyl alkyl-phosphonothiolic acid (VX). Toxicologically these agents are similar to many of the commercially available organophosphorous pesticides, such as parathion, TEPP or tetram and carbamates.

The physiological action of all OPs results from their inhibition of AChE. Organophosphorus compounds possess a P=O group that allows the same reaction to occur with AChE as between AChE and the C=O group of ACh. However, instead of an acetylated enzyme, now

a phosphylated enzyme is formed (see Fig. 3). This binding progresses in 4 different stages (Aldridge, 1953; Reiner and Aldridge, 1967).
The first stage is the formation of the reversible Michaelis-type complex. The next stage is the acetylation step that results in either phosphorylation by organophosphorus compounds or carbamylation by carbamates (Fig. 3). In this form the enzyme can reactivate spontaneously, by hydrolysis, to yield the original catalytic esterase, called the third stage. When in case of the phosphorylated enzyme a group (X) leaves the enzyme leaving a phosphorous behind in the AChE then the binding has become covalent and the enzyme is inactivated (Fig. 3). Depending on the type of OP that has bound onto the enzyme, the enzyme can reactivate by hydrolysis (Fig. 4). This reactivation occurs very slowly but can be reinforced by an oxime (Fig.4). When in case of the phosphorylated enzyme an alkyl group (R') leaves the enzyme then the enzyme can not be reactivated and is now irreversibly changed; this reaction is called the ageing reaction (Fig. 4) (Hobbiger, 1956; Berends *et al.*, 1959; Fleisher and Harris, 1965).

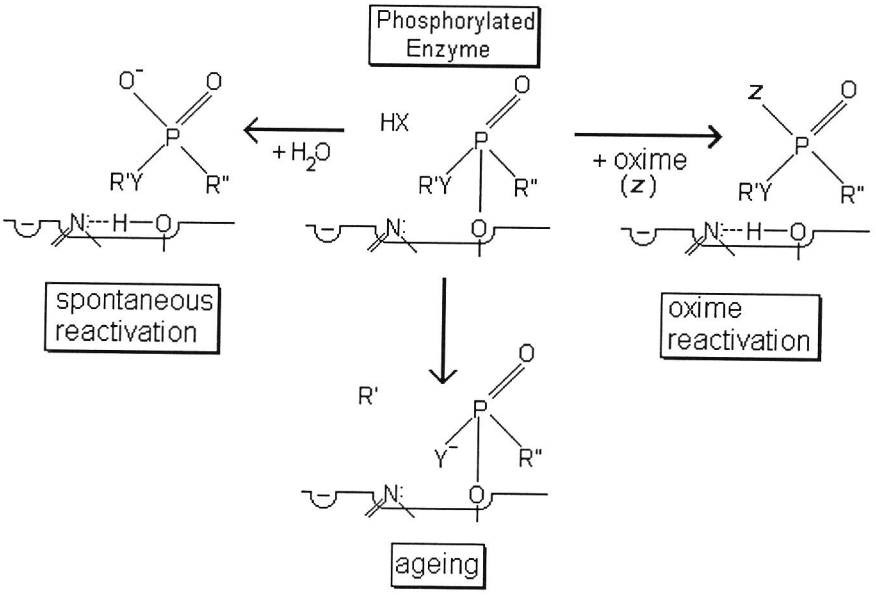


FIG. 4: Steps involved in the reactivation (spontaneous by hydrolysis or by an oxime) or ageing of phosphylated enzym based upon previous kinetic, spectroscopic, and chemical modification studies.

Current treatment regime of OP intoxication

Adequate protection against exposure to OPs can be achieved by wearing protective gear and a gas mask. Continuously this equipment must be tested for their protective capacity against newly developed OPs. In case a soldier is not sufficiently protected his body will get exposed and therapeutic measures should be taken. The cardinal principles of therapy for OP intoxication are enzyme reactivation with an oxime, parasympathetic blockage (with

atropine), decontamination, and ventilation. As quickly as possible the contaminated skin area should be decontaminated with a decontamination powder. These compounds inactivate the nerve agent before it can enter the body. One of these tools is the hypochlorite ion. But the most important and effective decontamination is the physical removal from the skin. When the nerve agent has entered the circulation then therapy should be aimed at reactivation of AChE before the ageing reaction has occurred (Berends, 1964; Fleisher, 1965). Hydrolytic regeneration of acetylated AChE can be obtained with an oxime (Wilson, 1954). Wilson found that this could be obtained by a molecule containing both a quaternary N-atom and an oxime group, spaced at an appropriate distance. When ageing of AChE has occurred no reactivation of AChE can be obtained. In such a case only a therapy with atropine is possible. Atropine prevents the actions of the accumulated acetylcholine by blocking the ACh receptor (Koplovitz, 1995). Not only due to the rapid ageing of the phosphylated AChE and the poor reactivatability of non-aged enzyme (oxime-resistancy), but also the predominant effects on the CNS, and the persistence in "depots" in the body make the nerve agent soman one of the most dangerous and difficult to treat OP intoxication (Wolthuis, 1981). For these reasons pretreatment (prophylaxis) has been considered in addition to treatment.

Pretreatment

Prerequisites for a successful pretreatment

A successful pretreatment to be used against OP intoxication optimally should fulfil 3 conditions:

1) it should offer a high protection rate,
2) it should not cause side effects, and
3) it should protect against post-intoxication incapacitation.

Protection against lethality

A pretreatment against OP intoxication is aimed at protecting a fraction of the AChE from irreversible binding by the OP, thereby allowing sufficient active AChE to be present in the body. Therefore, successful pretreatment can be achieved by compounds that bind to AChE and spontaneously hydrolyse from the enzyme. Commonly such compounds are called reversible inhibitors. This is a seemingly paradoxical situation of 2 compounds with the same mechanism of toxicity in which one protects against the toxicity of the other. However, due to the reversible binding of the pretreatment compound, like carbamates such as pyridostigmine and physostigmine, with AChE, AChE activity may return fast enough to prevent lethality following OP intoxication (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978; Harris *et al.*, 1980). This strategy should offer a high protective ratio expressed as the LD_{50} of OP in pretreated animals divided by LD_{50} of OP in unpretreated animals. Compounds that show reversible binding to AChE are carbamates (currently used) and reversible OP inhibitors like the insecticide tetraethyl pyrophosphate (TEPP) (Clermont, 1854) or phosphoramidates. At present, no phosphoramidate with a high protective ratio has been identified (Philippens, Chapter 3; Melchers *et al.*, 1994; Langenberg *et al.*, 1996). The drawback of TEPP was the necessity of an oxime to reactivate the enzyme after intoxication.

Side effects

Side effects of drugs when given to healthy persons should be minimised as much as possible, in particular when such a pretreatment should have to be taken during several weeks. Since pretreatment against OP intoxication should also inhibit AChE in the brain, the accumulation of ACh could lead to unwanted side effects like reaction, concentration, memory, and learning disabilities. The side effects can be counteracted by a cholinolytic that binds to the post-synaptic receptor without affecting it and prevents the binding of the transmitter ACh. The cholinolytics used are: atropine sulphate and scopolamine (Koplovitz, 1995; Leadbeater *et al.*, 1985). These drugs have proven to be able to counteract effects on autonomic effector cells and on cortical and subcortical sites in the CNS, where the receptors are largely of the muscarinic type (Berry and Davies, 1970).

Post-intoxication incapacitation

Most regimens are effective in preventing lethality from OP intoxication but do not prevent toxic effects and incapacitation. Incapacitation due to the overstimulation of the peripheral ACh receptors leads to symptoms such as abdominal cramps, a decrease in heart rate, hypersalivation, urinary incontinence, muscle weakness, fasciculations, diarrhoea, and blurred vision. Incapacitating effects resulting from overstimulation of central ACh receptors lead to convulsions. Severe convulsions induce irreversible brain damage in cholinergic areas. In case chemical weapons are used during wartime, exposure to the nerve agent around the centre of an explosion will almost certainly be lethal when no adequate protection is present. However, the nerve gas will not only contaminate the area of the explosion but also the surroundings. In the surroundings the concentration of the nerve gas will be lower and, therefore, only moderate to light intoxication might occur and certainly forms a risk factor. Due to this type of exposure in this area people may suffer from post-intoxication incapacitation.

Current pretreatment

Currently pyridostigmine bromide (Fig. 5) is used as a pretreatment against OP intoxication in combination with a therapy consisting of an oxime, atropine, and an anticonvulsant (diazepam). Pyridostigmine is also used clinically in the treatment of glaucoma.

Protection against lethality

Pyridostigmine is a carbamate that binds to the enzyme AChE in a reversible manner (Watts and Wilkinson, 1977). In Table 2 it is shown that pyridostigmine in combination with a post intoxication therapy protects effectively against lethality in a number of species (Gordon, 1978; Leadbeater, 1985; Dirnhuber *et al.*, 1979). However, pyridostigmine alone did not protect against the toxicant (Gordon *et al.*, 1978).

Side effects

During the Operation Desert Storm soldiers were given pyridostigmine in a blister pack containing twenty-one 30-mg pyridostigmine tablets. Each soldier received instructions to take one tablet every 8 hours. This dose of pyridostigmine was expected not to show undesirable cholinergic effects. Nevertheless, peripheral and central side effects were

recorded (Keeler *et al.*, 1991). The central effects could be the result of stress. First of all stress itself could be an important factor, however, stress also enhances the passage of pyridostigmine across the blood-brain barrier (Friedman, 1996). In the Operation Desert Storm nine cases of pyridostigmine self-poisoning were encountered. These individuals only suffered from peripheral cholinergic symptoms such as abdominal cramps, diarrhea, hypersalivation, blurred vision etc, whereas no effects on the central nervous system were observed (Almog, 1991).

Post-intoxication incapacitation

The structure of pyridostigmine contains a quaternary nitrogen atom (see Fig.5). For this reason pyridostigmine hardly penetrates the brain and will not inhibit brain-AChE. A single im injection of pyridostigmine (131 μg/kg) caused 58.5 % inhibition of blood AChE and no inhibition of brain AChE, while a tertiary derivative 3-(N,N-dimethylcarbamyloxy)-1-methyl-Δ^3-tetrahydropyridine (THP) caused, given at a comparable dose, 30.0 % inhibition of blood

TABLE 2

Protective ratios against OP intoxications after prophylactic treatment with pyridostigmine (PYR) in different animal species

Species	Dose PYR (mg/kg)	Prophylactic interval (min)	Protective ratio			
			Tabun	Sarin	Soman	VX
Rat	0.075(im)	20 +	1.2	1.5	1.7	5.0
Rabbit	0.1 (im)	30 +	4.6	27.0	2.7	5.0
Guinea pig	0.1 (im)	30 +	22.0	21.5	5.3	17.9
Guinea pig	0.1 (im)	30 *	17.0	3.8	5.5	2.9
Marmoset monkey	0.2 (iv)	10 **			15.0	
Rhesus monkey	0.2 (iv)	15 **			28.0	

Protective ratio: LD_{50} of OP in pretreated animals / LD_{50} of OP in nonpretreated animals.

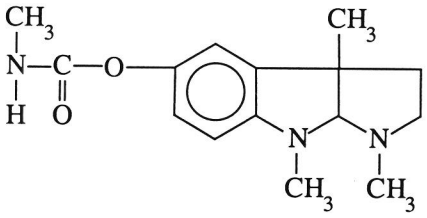
\+ oxime P2S (30 mg/kg) and atropine sulphate (17.4 mg/kg) (im) were given therapeutically 1 min after OP intoxication

* atropine sulphate (17.4 mg/kg) (im) was given therapeutically 1 min after OP intoxication

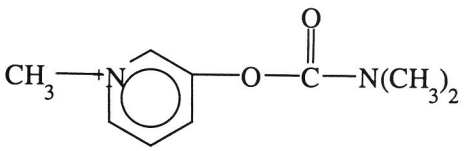
** atropine sulphate (4 mg/kg) (im) was given therapeutically 1 min after OP intoxication

(data collected from Gordon *et al.*, 1978 and Dirnhuber *et al.*, 1979)

AChE and 25.3 % inhibition of brain AChE (Ray, 1991). This is in accordance with findings in behavioural studies: no central effects were found after pyridostigmine compared with low dose levels of physostigmine or soman (Wolthuis *et al.*, 1995). Therefore, pyridostigmine does not protect the CNS against the toxic influences of OPs.



Physostigmine



Pyridostigmine

FIG. 5: Structural formulae of physostigmine and pyridostigmine

Physostigmine as a replacement for pyridostigmine

Physostigmine (Fig. 5) is derived from the seeds of the calabar bean Physostigma venenosum growing in West Africa. Daniell, a British medical officer, brought this bean to Engeland in 1840. It is an alkaloid that inhibits AChE. This inhibition was first established in 1946. It was shown that after pretreatment of cats with a small dose of eserine (PHY) the animals could withstand several times the LD_{50} of diisopropylphosphorofluoridate (DFP) (Koster, 1946). Physostigmine has also been used clinically. The first therapeutic use of physostigmine was in the treatment of glaucoma by Laqueur in 1877. Another therapeutic application was the improvement of memory functions in patients with Alzheimer's disease (Muramoto *et al.*, 1984; Whitehouse, 1993; Thal *et al.*, 1983). Furthermore, physostigmine can reverse the toxic effects associated with anticholinergic poisoning (Rumack, 1973) or with an overdosage of other drugs (Nattel, 1979). In the latter study physostigmine restored vital functions until the drugs were eliminated from the body. Physostigmine is used to compensate overdosage of tricyclic antidepressants (Di Liberti *et al.*, 1975; Larson *et al.*, 1977), morphine (Weinstock *et al.*, 1981) and benzodiazepines (Bernards, 1973). Because the carbamate physostigmine, like pyridostigmine, reversibly inhibits AChE, it could replace pyridostigmine as a prophylactic treatment. For being an effective pretreatment physostigmine should meet the earlier mentioned three conditions:

Protection against lethality

It has been shown that physostigmine pretreatment is effective against OP poisoning: a significant protection against lethality after sarin and soman intoxication has been reported (Leadbeater *et al.*, 1985). After an acute physostigmine pretreatment a protective ratio against lethality in guinea pigs of 14.4 after soman poisoning followed by a post intoxication therapy was reported and of 2.5 without post-intoxication therapy. In another study subchronic physostigmine (0.0048 mg/hr) pretreatment for 6 days prior to LD_{99} soman offered a survival rate of 4/8 and when combined with scopolamine (0.0019 mg/hr) of 8/8 (Wetherell, 1994).

Side effects

In contrast to pyridostigmine the molecular structure of physostigmine contains a tertiary nitrogen atom, that allows this molecule to pass the blood-brain barrier (see Fig 1) and exert effects at both peripheral and central cholinoceptive sites. When taken for a prolonged period unwanted side effects may be expected to develop. In studies of Alzheimer's disease, a lot of research has been carried out with respect to physostigmine. Focusing on the clinical symptoms, physostigmine leads to dose dependent central and peripheral effects like hypersalivation, hypothermia, miosis and tremors (Yoshida and Suzuki, 1993). Subchronic physostigmine (0.12 mg/kg/hr) in the guinea pig, leading to blood AChE inhibition of 20-40% and brain AChE inhibition of 20%, did not affect the body temperature and water consumption and induced no tremors (Lim *et al.*, 1988a).

Also effects in behavioural test systems were described after physostigmine: in an operant conditioning chamber a decrease in performance was found after physostigmine (0.4 mg/kg sc in the rat) (Genovese *et al.*, 1990). The antimuscarinic scopolamine was able to antagonise this effect completely. In cases of subchronic physostigmine treatment, leading to blood AChE inhibition of 42%, no effects on the motor performance measured by an accelerating rota-rod was found (Harris *et al.*, 1989). Neurophysiological physostigmine (1 mg/kg) modulated the visual evoked potential (VEP) of the rat in a behaviour-related manner: all the VEP peaks changed conformable with a high arousal behavioural states (Bringmann, 1994).

Post-intoxication incapacitation

As mentioned before, physostigmine easily penetrates the brain and, therefore, should protect the CNS against the toxic influences of OPs. Following soman intoxication guinea pigs exhibited signs of OP poisoning like hyperactivity, chewing, tremors, prostration, and salivation. Guinea pigs pretreated with subchronic physostigmine (offering a blood AChE inhibition of 26%) showed no obvious signs of poisoning at 4 h post-intoxication, whereas in the guinea pigs pretreated with the combination of subchronic physostigmine and scopolamine the signs already had disappeared 2 h post-intoxication (Wetherell, 1994). The protective ratio in guinea pigs against post soman intoxication incapacitation, measured by gross motor performance in a swimming test, was found to be 2.0 after physostigmine pre-treatment and 1.0 after pyridostigmine pretreatment (both in a comparable dose leading to a peak erythrocyte AChE inhibition of 70%) (Leadbeater *et al.*, 1985). This is in accordance with another study in rats: subchronic physostigmine pretreatment protected significantly better against soman induced incapacitation than subchronic pyridostigmine pretreatment (Miller *et al.*,1993).

Aims of this thesis:

As outlined in the previous paragraphs, the current pretreatment regimen for OP intoxication consisting of oral administration of pyridostigmine is far from ideal. Therefore, this pretreatment should be improved. For the time being, physostigmine seems a promising alternative. Before the pretreatment scenario with pyridostigmine can be replaced by physostigmine some questions have to be addressed:

Is physostigmine a better alternative for pyridostigmine pretreatment regarding the prerequisites for a successful pretreatment?

Protection against lethality

As mentioned before physostigmine pretreatment in combination with a post-intoxication therapy was found to be effective against OP poisoning (Leadbeater *et al.*, 1985). This compound has also proved to be more effective then pyridostigmine in preventing toxic effects after soman poisoning in guinea pigs and rats (Solana *et al.*, 1990; Miller *et al.*,1993). In this thesis the efficacy of different pretreatment protocols in protecting animals against lethality after soman poisoning were investigated. In Chapter 5 the protection of four different pretreatment regimes followed by a post-intoxication therapy of atropine against $3xLD_{50}$ soman intoxication was tested and compared in the guinea pig. The pretreatment scenarios consisted of: a single dose of physostigmine or pyridostigmine combined with scopolamine given 30 minutes prior to soman, or subchronic physostigmine alone or in combination with scopolamine for 10 days prior to soman. In Chapter 5 the protection of subchronic physostigmine pretreatment in combination with a post-intoxication therapy of atropine against $2xLD_{50}$ soman intoxication was tested in the marmoset monkey and compared with the efficacy of the post intoxication therapy of atropine in nonpretreated animals.

Side effects

Most side effects of physostigmine pretreatment can be expected to be centrally mediated effects. Depending on the affected brain areas, these effects can induce changes in different types of behaviour. For this reason suitable behavioural test systems were developed to elucidate the severity of side effects during short-term and long-term pretreatment regimens. In Chapter 2 newly developed test systems for the guinea pig and the marmoset monkey are described. For the guinea pig an active avoidance task, using a sound signal as a conditioned stimulus and an air stream ruffling their fur as an unconditioned stimulus, was developed. The sensitivity of this task for anti-cholinergic drugs was tested with physostigmine and the combination of physostigmine with scopolamine. For the marmoset monkey two test systems were developed: 1) a motor activity task that was evaluated with 2 drugs that should affect the motor activity, and 2) a robot-guided hand-eye co-ordination task in which the sensitivity was tested by different ChE inhibitors. Other read-out systems for both animal species used in this thesis were the startle response and for measuring neurophysiological side effects EEG and the visual evoked response (VER) registration.

In Chapter 3 the anti-cholinergic side effects of acute physostigmine were compared with another AChE inhibitor in the guinea pig, i.e., ethyl para-nitrophenyl phosphoramidate (PNF). The mechanism of the effects on the startle response that could not be counteracted by scopolamine and therefore presumably were not caused by AChE inhibition were further examined and described in Chapter 4. It is known that physostigmine can affect receptors in a

direct manner (Albuquerque *et al.*, 1984; Albuquerque *et al.*, 1988; Sherby *et al.*, 1984). The ED_{50} of physostigmines' agonism at the nicotinergic receptor even appears to be lower than its IC_{50} of AChE inhibition (Albuquerque *et al.*, 1988). Studying the mechanisms of the effects of physostigmine will learn us more about its protective effect.
Because a single injection of physostigmine as pretreatment is not a very realistic approach, a more chronic application of physostigmine as pretreatment was evaluated. The side effects of subchronic pretreatment with osmotic pumps are described in Chapter 5 for the guinea pig and in Chapter 6 for the marmoset monkey.

Post-intoxication incapacitation
Post-intoxication incapacitation can be measured by observing the clinical signs (Wetherell, 1994; Miller *et al.*, 1993; Solana *et al.*,1990). But, when these signs have disappeared the animals may still suffer from the OP intoxication. These incapacitating effects may influence the soldier's performance and therefore need further evaluation. In Chapters 6 and 7 animal studies are reported that measure post intoxication incapacitation objectively by using behavioural and neurophysiological read-out systems.

What should be the ideal regime for pretreatment with respect to OP intoxication?

Because physostigmine can easily penetrate into the brain, the risk of side effects will be high. To abolish these undesired side effects, the muscarinic antagonist scopolamine can be added to the pretreatment (Koplovitz *et al.*, 1995; Leadbeater *et al.*, 1985). This results in multiple pharmacology. The question addressed in this thesis was: is additional therapy with scopolamine necessary to antagonise side effects and improve the efficacy of the pretreatment? And does an adequate pretreatment regimen offer sufficient protection by itself? This last question has to be addressed because in case of nerve gas exposure in a war situation the soldiers have to inject themselves with a post-intoxication therapy. One can envisage situations that soldiers are deprived of or are not capable of using an auto-injector containing therapy. In Chapter 7 the efficacy concerning the survival and post-intoxication incapacitation of subchronic physostigmine pretreatment with or without the addition of scopolamine was tested in combination with a post-intoxication therapy of atropine or without an additional therapy.

How predictive are experimental data in guinea pigs and marmoset monkeys on pretreatment protocols for the application in man?

In the past, research with anti-ChE was performed in the rat (Wolthuis and Vanwersch, 1984). In contrast to man, rats have high amounts of carboxylesterase in their blood. These esterases can act as scavengers for the anti-ChE drugs. For this reason the rat is not a suitable model for man. Therefore, animals that have relatively low blood carboxylesterase levels are preferable models, i.e., guinea pig (Maxwell *et al.*, 1987) and marmoset monkey. The marmoset also proved to be a good model for man to measure enzyme reactivity after soman intoxication (Van Helden *et al.*, 1983). Furthermore, toxicokinetic studies reported that the concentration time profile of soman in guinea pigs resembles that of the marmoset monkey more closely than that of the rat (Benschop and De Jong, 1991). For these reasons the guinea pig and the marmoset monkey have been used for experimental work presented in this thesis. The predictive relevance of these data for man will be discussed in Chapter 8.

2

Behavioural test systems

2.1

Active avoidance behaviour in guinea pigs: Effects of physostigmine and scopolamine

Ingrid H.C.H.M. Philippens, Bert P.C. Melchers and Otto L. Wolthuis

TNO Medical Biological Laboratory (TNO-MBL), Research Group Pharmacology, Rijswijk ZH, The Netherlands.

Behavioural training of guinea pigs by conventional methods, such as used for rats and mice, appears difficult. Hence, only a few behavioural experiments with guinea pigs have been described in the literature. An active avoidance technique in an automated two-way shuttlebox is described, using sound as a conditioned (CS) and a tactile stimulus (a stream of air ruffling their fur) as an unconditioned (UCS) stimulus. Acquisition is fairly rapid and reproducible. Doses of physostigmine that caused moderate blood-acetylcholinesterase inhibition induced dose-dependent performance decrements. These decrements were counteracted by a sign-free dose of scopolamine.

Pharmacol Biochem Behav 42:285-289, 1992

Introduction

Administration of drugs or exposure to neurotoxic agents may disturb behaviour. For the detection of such behavioural effects usually rats or mice are employed. However, for experiments on the pretreatment and therapy of intoxications with organophosphate cholinesterase (ChE) inhibitors these species may have some draw-backs, in particular for in vivo studies. In contrast to human blood, the blood of rats and mice contains large amounts of carboxylesterases, which act as scavengers for ChE inhibitors. This difference may hamper extrapolation to man of data obtained with these rodent species. Hence, several authors investigating ChE-inhibitors prefer the use of guinea pigs, animals that have only small concentrations of carboxylesterase present in their blood.

In some cases it is desirable to measure the behavioural effects of ChE-inhibitors, for example, when testing carbamates for their potential use as pretreatment agents against organophosphate poisoning (Leadbeater *et al.*, 1985). Unfortunately, reports on behavioural experiments in guinea pigs are rare. Upon a quick computer-search, only nine papers were found; two on psychophysical experiments for auditory thresholds (Spencer and Schaumberg, 1980; Stebbins and Moody, 1979), one on open-field behaviour (Tobach and Gold, 1966), one on motor performance in swimming test (Rylands, 1982), one on water maze (Smart and Adlard, 1974) and four on foot-shock-motivated shuttlebox acquisition and performance (Ashton and Werbrouck, 1991; Clincke and Wauquier, 1984a; Clincke and Wauquier, 1984b; Sansone and Bovet, 1970), three of which originate from the same laboratory. Pilot experiments in our own laboratory resulted in erratic behaviour in open-field tests and lack of reproducible performance and freezing of the animals in foot-shock-motivated shuttlebox avoidance conditioning, even at low scrambled foot-shock levels of 100-200 μA (constant-current principle). In an investigation of different stimuli it was observed that a stream of air, ruffling the fur of the guinea pig, might be used as an unconditioned stimulus in a shuttlebox test. The present results show that this is indeed the case and further demonstrate that the effects of physostigmine alone or in combination with scopolamine can be measured in a sensitive and reproducible fashion.

Method

Animals

Dunkin-Hartley albino guinea pigs CrL:(HA)BR (Charles River) with a starting body weight of 275-325 g were used. All animals were male and experimentally naive.

Apparatus and Procedures

The two-way shuttlebox consists of two equal compartments (23x23x23 cm) with a linoleum floor connected by a gate, guarded on each side by an infrared beam, through which the animal may cross from one compartment to the other. Per day, each animal received one session of 20 trials, during which the animal had to learn to avoid the UCS by moving into the other compartment within 10 seconds after the sound signal [conditioned stimulus (CS)] had been turned on. The sound signal consists of white noise passing through a bandpass filter with a centre frequency of 10 kHz and a slope of 12 db/oct, at a level of 65 db measured in the middle of each cage. The sound signal stops when the guinea pig has passed through the gate. When the animal fails to avoid, a stream of air (6,250 cm^3/sec, air tube diameter 1 cm) directed into the compartment in which the animal is present is turned on and stops when the animal has escaped into the other compartment. The intertrial interval is 25 s (± 20% random). When the guinea pig fails to escape, the air stream is turned off after 20 s. After the animals had reached their criterion, which was 80% or more correct avoidance responses, the drugs were injected s.c. Two experiments were carried out. In the first experiment the animals were injected with saline (1 ml/kg) or physostigmine in different doses (0.3, 0.6, or 1.2 mg/kg) to investigate the sensitivity of this test for a centrally active carbamate. In the second experiment, the two highest doses of physostigmine were injected alone or in combination with scopolamine (100 µg/kg). Control animals received saline (1 ml/kg) in combination with scopolamine (100 µg/kg). In both experiments, animals were tested 0.5 and 24 hours after injection. Avoidances, escapes and intertrial responses were detected by the sequential interruption of the infrared lightbeams and were processed by a Hewlett-Packard Vectra personal computer, programmed in Pascal. In a separate group of unanaesthesized animals blood samples (5 µl) were drained from their ear veins immediately before and 10, 30, and 60 min after injection of the same physostigmine doses used in Experiment 1, that is, 0.3, 0.6 or 1.2 mg/kg sc Acetylcholinesterase (AChE)-activity was determined as follows: Blood samples (5 µl) were immediately mixed with 1% saponin (BDH, Poole, England) and frozen in liquid N_2. After appropriate dilution samples were assayed for acetylcholinesterase activity using a radiometric method (Johnson and Russell, 1975); the acetylcholine (ACh) end concentration used was 12 µM; [^{3}H]ACh iodide (NEN, Dreieich, Germany) was diluted to a specific activity of 600 $MBq.mmol^{-1}$. To limit decarbamoylation of physostigmine-inhibited-AChE, samples were kept at 0°C until they were incubated with [^{3}H]ACh for 15 min at 20°C. Ethopropazine (2.5 µM; Sigma Chemical Co., St Louis, MO) was used as specific inhibitor of butylcholinesterase (BuChE). AChE from electric eel was used as a reference.

Chemicals

All chemicals were obtained commercially. Solutions were freshly prepared before use.

Statistics

The multiple t-test of Welch was used. When the term significant is used, this means $p<0.05$ (two tailed).

Results

General

Upon leaving the tube supplying the air stream, a hissing sound is produced that might act as an UCS. In a pilot experiment, by aiming the air tube in another direction it could be established that this hissing sound did not act as an UCS. During the experiments, performance of the animals was continuously monitored by color TV. In contrast with earlier experiences with foot-shock, freezing did not occur.

Intertrial responses (ITRs), usually taken as a measure of the activity levels of the animals, increased during training and varied largely between animals. For example, for all animals in Experiment 1 ranges of ITRs were 0 -> 5 in Session 1, 0 -> 15 before injection in Session 7 (with one exception making 40 ITRs), and 0 -> 17 after injection during Session 8, again with one exception making 27 ITRs (not the same animal that made 40 ITRs during Session 7). There were no significant differences between the averaged ITRs of the groups either before or after the injections.

In preliminary experiments, it was found (not shown) that acquisition was not faster when two sessions (each of 20 trials, one in the morning, one in the afternoon) were given instead of only one session of 20 trials per day.

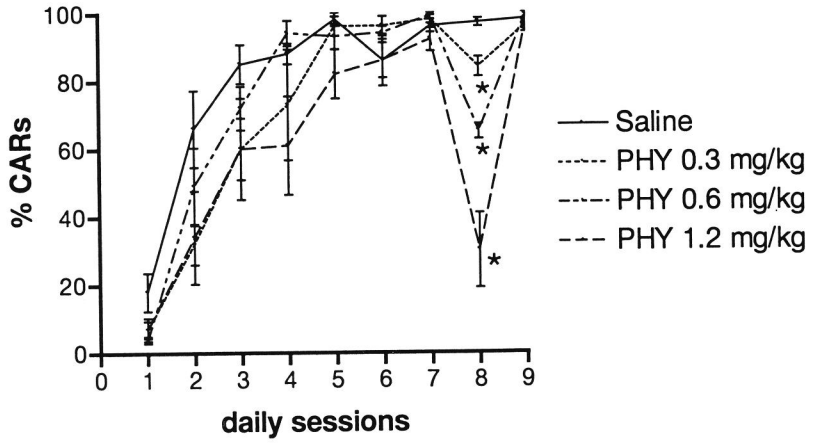


FIG. 1. Mean (± SEM) acquisition rates and performance of guinea pigs. Four groups of guinea pigs (n=5/group) were trained in a two-way shuttlebox (CS, sound; UCS, airstream; 1 session/day, 20 trials/session). On day 8, different doses of physostigmine (PHY) were sc injected and 30 min later another session started. It can be seen that acquisition rates of the four groups is very similar. Performance levels at day 7, judged by the % correct avoidance responses (CARs) are >90%. Different doses of physostigmine cause dose-dependent performance decrements. After 24 h performance has returned to preinjection values.

*Significantly different from each other and from controls. inj., injection.

Experiment 1

In Fig. 1, it can be seen that even with small groups of guinea pigs (n=5/group) comparable acquisition rates may be obtained. Upon reaching a high level of performance, dose-dependent decrements of performance were found 30 min after sc injection of different doses of physostigmine. Twenty-four hours later the animals had returned to their pre-injection performance level. The changes in blood-AChE activity following these doses of physostigmine were determined in parallel groups of animals; the results (Fig. 2) show that there was a weak dose-dependent inhibition of blood AChE. The average values all ranged between 41-66 % inhibition of AChE.

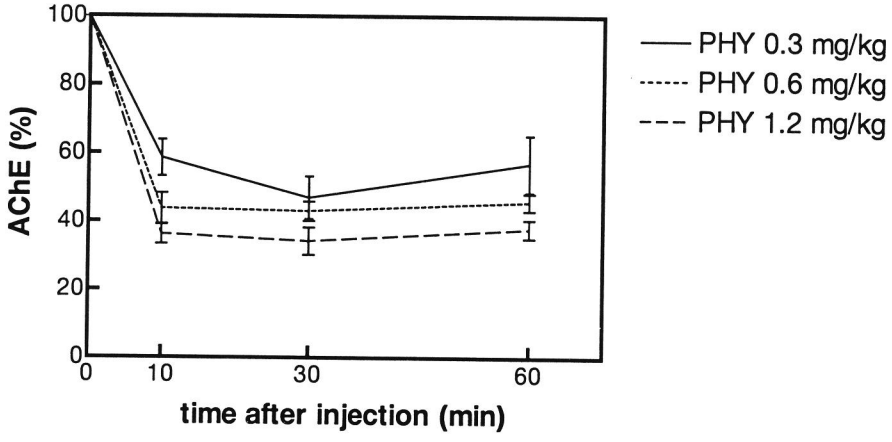


FIG. 2. Effect of single sc injections with physostigmine (PHY) in doses of 0.3, 0.6 or 1.2 mg/kg on the mean (± SEM) AChE-activity in blood from unanaesthesized guinea pigs. Values (mean ± SEM) are expressed as percentages of the preinjection values.

Experiment 2

In Fig. 3, the combined results are shown of two independent, procedurally identical experiments. Both experiments were performed with four animals/treatment group; the results were virtually identical and therefore combined. In essence, part of the experiment, that is, groups treated with 0.6 or 1.2 mg/kg physostigmine, are a repetition of Experiment 1, providing very similar results. It can also be seen in Fig. 2 that scopolamine (100 µg/kg, sc) by itself causes no significant effect. It counteracts the performance decrement caused by physostigmine 0.6 mg/kg to practically pre-injection values and improves the performance decrement caused by 1.2 mg/kg physostigmine significantly. In all cases, performance was back to preinjection levels after 24h.

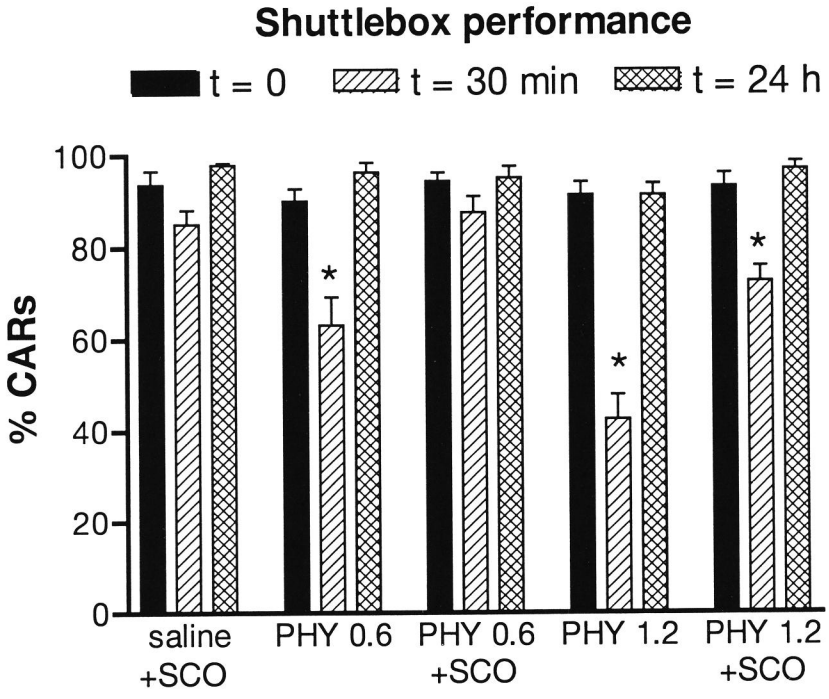


FIG. 3. Shuttlebox performance (mean ± SEM) of guinea pigs 24 h before, 30 min, and 24 h after sc injection of either 0.6 or 1.2 mg/kg physostigmine (PHY) alone or in combination with 100 µg/kg scopolamine sc It can been seen that this dose of scopolamine has no significant effect, whereas it effectively counteracts the behavioral decrements caused by physostigmine.
*Significantly different from performance 24 h before and 24 h after injection.

Discussion

The levels of carboxylesterases in blood of guinea pigs are lower than in mice and rats and much closer to those in human and nonhuman primates. These blood carboxylesterases act as scavengers for organophosphates (OPs) and their blood levels may be the most important determinant for the large differences in OP toxicity across species (Boskovic, 1979; Maxwell *et al.*, 1987). Hence, the guinea pig is a suitable experimental animal to investigate prophylactic, pretreatment, and therapeutic measures against OP intoxication, particularly for in vivo studies.
An essential step in the evaluation of the efficacy of these measures is the assessment of the behavioural incapacitation that may be induced by a prophylaxis or pretreatment, or the incapacitation that remains after therapy. However, as mentioned earlier, reports on the assessment of guinea pig behaviour are scarce and the results of several attempts in our laboratory left much to be desired. After investigating different stimuli, an air stream was subsequently applied as an unconditioned stimulus in a two-way shuttlebox design. The results

are shown in Fig. 1: Acquisition is fairly rapid and reproducible between four groups of animals, variability in performance between animals is acceptable, and performance levels reached in 6-7 days was close to 100% avoidances. The administration of different low doses of physostigmine demonstrates that dose-response effects can be reliably obtained. It is not likely that this was due to deterioration of the condition of the animals; upon close observation, no overt behavioural changes were detected, whereas their level of activity, judged by the number of ITRs, was not significantly changed.

A carbamate was chosen because carbamate pretreatment protects against OP intoxication (Gordon *et al.*, 1978); pyridostigmine pretreatment has now been adopted by several nations. However, pyridostigmine as a quaternary compound does not readily penetrate the CNS, and does not protect against post-intoxication behavioural incapacitation. The tertiary carbamate physostigmine also protects against OP intoxication and since it passes the blood-brain barrier it should offer some protection of the CNS against an OP intoxication and thereby counteract the postintoxication incapacitation. However, at the dose levels of physostigmine required to provide protection against OP intoxication (causing 40-70% carbamoylation of blood AChE) physostigmine itself may induce behaviourally incapacitating effects, presumably due to the fact that it penetrates the brain. It will be clear that incapacitation induced by a pretreatment is unacceptable. Hence, Leadbeater *et al.* (for a full discussion of these problems see Leadbeater *et al.*, 1985) attempted to counteract these behaviourally incapacitating effects of physostigmine pretreatment by combining physostigmine with a small dose of the cholinolytic scopolamine (see also Genovese *et al.*, 1990). This combination was effective in a swim test. However, this test measures gross motor performance and it is obvious that other behavioural tests should be applied to investigate the efficacy of the combination of these two drugs. It was for this reason that the second experiment was carried out. As seen in Fig. 3, the addition of a sign-free dose of scopolamine indeed counteracts the performance decrements caused by physostigmine. The results obtained so far indicate that for these and other purposes the behavioural method presented here may be worthwhile considering. The results of the AChE measurements in blood (Fig. 2) demonstrate that the method is sensitive enough to measure the behavioural effects of protective doses of physostigmine that cause a 41-66 % inhibition of blood AChE.

Acknowledgement

We are grateful to dr. R.W. Busker and J.J. Zijlstra, who performed the AChE determinations.

2.2

Hand-eye coordination: Low doses of ChE-inhibitors in marmosets

Otto L. Wolthuis, Bas Groen, Raymond A.P. Vanwersch, Ingrid H.C.H.M. Philippens, Ruud W. Busker and Herma J. van der Wiel

TNO Medical Biological Laboratory (TNO-MBL), Research Group Pharmacology, Rijswijk ZH, The Netherlands.

Based on earlier results in rats, the effects of single low doses of soman, sarin, physostigmine and pyridostigmine were tested on the performance of a hand-eye coordination or a discrete two-choice visual discrimination task in marmosets (Callithrix jacchus). Total blood ChE-activity was measured before and after testing. In addition, effects on several physiological parameters (ECG, heart rate, blood pressure, respiratory minute volume and neuromuscular transmission) were measured in a separate group of anaesthetised marmosets, following a single injection of each of these four inhibitors at a dose-level that caused behavioural decrements.

The preliminary results obtained in the 80 marmosets that have been used so far in these experiments suggest that - as in rats - behavioural decrements induced by these inhibitors occur at dose-levels that cause no changes in overt behaviour, considerable total blood-CHE inhibition and no/hardly any physiological effects. Soman and physostigmine cause behavioural effects at lower doses than sarin and pyridostigmine, respectively. Moreover, performance of hand-eye co-ordination seems more susceptible to disruption by these inhibitors than visual discrimination.

Proc Med Def Biosc Rev 7-8:469-473, 1991

Introduction

Earlier results from our laboratory indicated that in rat organophosphate and carbamate cholinesterase inhibitors induced dose-dependent behavioural decrements at dose-levels far below those that cause overt effects (Wolthuis and Vanwersch, 1984).
Dose-wise, the minimal effective dose (MED) of soman was lower than that of sarin and the MED of physostigmine (PHY) was lower than that of pyridostigmine (PYR).
If applicable to humans, it is not unlikely that some of these agents may cause mental disturbances without physical signs or even without being noticed by the victim. The chance that these effects also occur in man increases if they can also be demonstrated in another species. On the basis of earlier experience (Van Helden *et al.*, 1983) the marmoset was chosen.

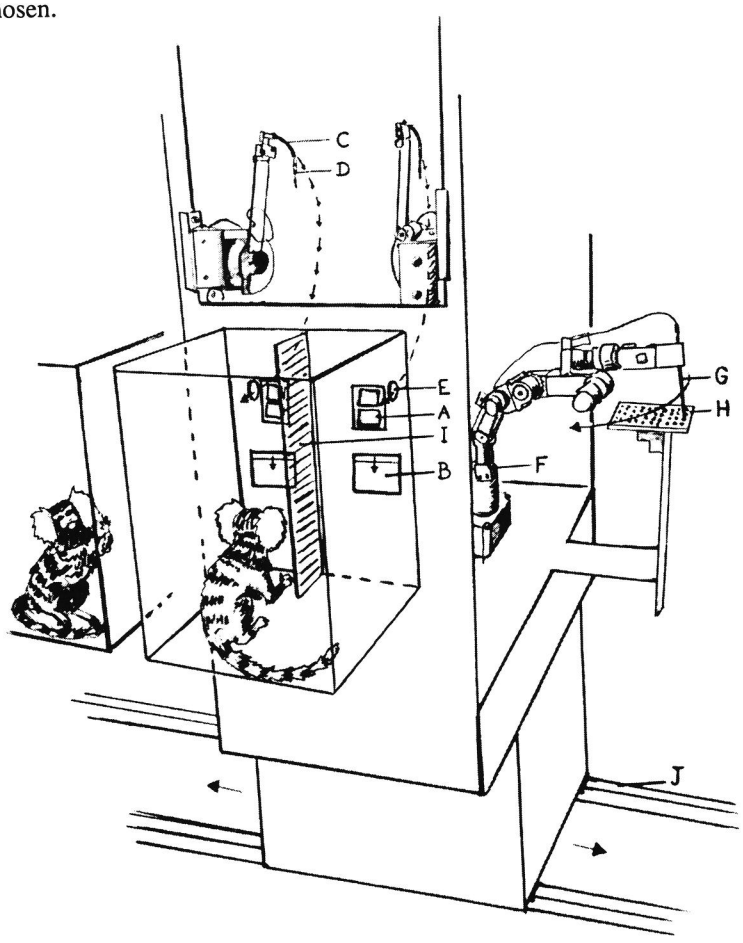


FIG.1.Schematic illustration of the robot-assisted behavioural test apparatus for measuring the hand-eye coordination in marmoset monkeys motivated by positive reinforcement. A, α -numerical display; B, window; C, handles; D, small chain attached to the handle; E, holes through which the handles are introduced into the cage; F, robot arm; G, suction tube; H, tray with marshmallow-like rewards; I, partition screen; J, rails.

Method

So far, 80 experimentally naive male and female marmosets between 1.5-5 years old have been tested. The experiments are not finished and the results should be considered preliminary.

Training and testing (see also Wolthuis *et al.*, 1991)

The apparatus is shown in Fig. 1. The robot-arm (F) is situated between two test panels on a rack that is transported on a rail-track (J) along a row of test cages with marmosets. In each of the daily 40 trials, by means of a suction tube (G), the robot-arm picks a little piece of a marshmallow-like reward from the tray (H).

Discrete trial two-choice discrimination task

For the discrete trial two-choice discrimination task the reward is transported behind the closed window-door (B). At random time intervals a brief tone is presented, the left or the right (in a quasi-random order) α-numerical display (A) switches "on" and on both sides handles (C) are introduced into the cage through the holes (E). The animal is trained to pull the handle at the side where the α-numerical display is illuminated, whereupon the window-door on that side of the partition (I) slides open and the animal can retrieve the reward. The α -numerical display switches "off", the handles are retracted, the window is closed and a new trial can start.

Hand-eye coordination task

For the hand-eye coordination task only one window is used: after a brief tone it slides open and the animal is trained to grab the reward from the suction tube while it is moved by the robot from right to left at the other side of the window at a speed of 10 cm/s.

For both tasks the main indicator of performance is the percentage of "hits", i.e. the number of times/40 trials x 100% that the animals retrieve the reward. Testing takes place in 8 sessions. On the second of these 8 sessions saline
was injected im (this was repeated after 2 days if this caused a substantial performance decrement) and on the fourth session the animals were injected im with saline, soman, sarin, physostigmine or pyridostigmine. Behavioural testing started 20 min after physostigmine and 30 min after the other compounds were injected. A heel-prick method was used to obtain a drop of blood for the determination of total ChE-activity by a modification of the Ellman method (Ellman *et al.*, 1961); several hours before and immediately after behavioural testing.

The lowest dose of each compound that caused behavioural changes was tested in ketamine anaesthetised animals (n = 3 per compound) for its pharmacological effects on the ECG, heart rate (HR) average blood pressure (avBP), neuromuscular transmission (NMT) and respiratory minute volume (RMV), with techniques that have been reported before (Van Helden *et al.*, 1983).

Results

The effects of the low doses of ChE-inhibitors on various physiological parameters is shown in Table 1. They are expressed as percentage change of the animal's own control value, taken before injection. The numbers of animals/treatment group are indicated on top of each bar. Except after physostigmine, total ChE-activity in blood was more than 40 % inhibited; for a major part this was due to inhibition of AChE, BuChE-inhibition was slight (not shown). Apart from a small effect of pyridostigmine on heart rate (highly variable; the 12 control values: 244 ± 0.4/min) and on blood pressure (fairly constant; control values: 76 ± 3.1) hardly any effects were detectable.

The effects on total blood-ChE and performance of a hand-eye or a visual discrimination task are shown in Fig. 2. The results are expressed as changes of these three parameters, i.e. the effects of a compound minus the effect of saline (injected two days before) in the same animal.

TABLE 1

Percentage change in physiological parameters of anaesthetised marmosets, 20 or 30 min after i.m injection of single doses of ChE-inhibitors.

Drug (µg/kg)	n	HR (± SEM)	AvBP (± SEM)	NMT (± SEM)	RMV (± SEM)	BLOOD-CHE total	BLOOD-CHE AChE
soman (1.75)	3	83 (3)	97 (3)	107 (12)	100 (0)	44 (6)	21 (2)
sarin (6.0)	3	94 (10)	97 (6)	107 (6)	98 (2)	55 (9)	14, 19 (n=2)
PHY (20)	3	109 (5)	86 (11)	96 (5)	98 (2)	81 (2)	79, 90 (n=2)
PYR (200)	3	75 (13)	78 (4)	93 (3)	102 (2)	45 (14)	14 (10)

Discussion

The behavioural part of these experiments is not finished. It is intended to have n=6 for each relevant dose-group. However, some preliminary conclusions are: 1) except for soman at 7 µg/kg (LD_{50} = 8.7 µg/kg) and sarin at 12.0 µg/kg (D'Mello and Duffy, 1985) none of the doses of these compounds cause any overt effect, 2) soman and physostigmine cause effects at lower doses than sarin and pyridostigmine, respectively, 3) hand-eye coordination is more sensitive than visual discrimination. Gross differences between males and females were not detected.

The combined results indicate that behavioural changes occur at dose levels that are substantially lower than those that cause symptoms or physiological effects.

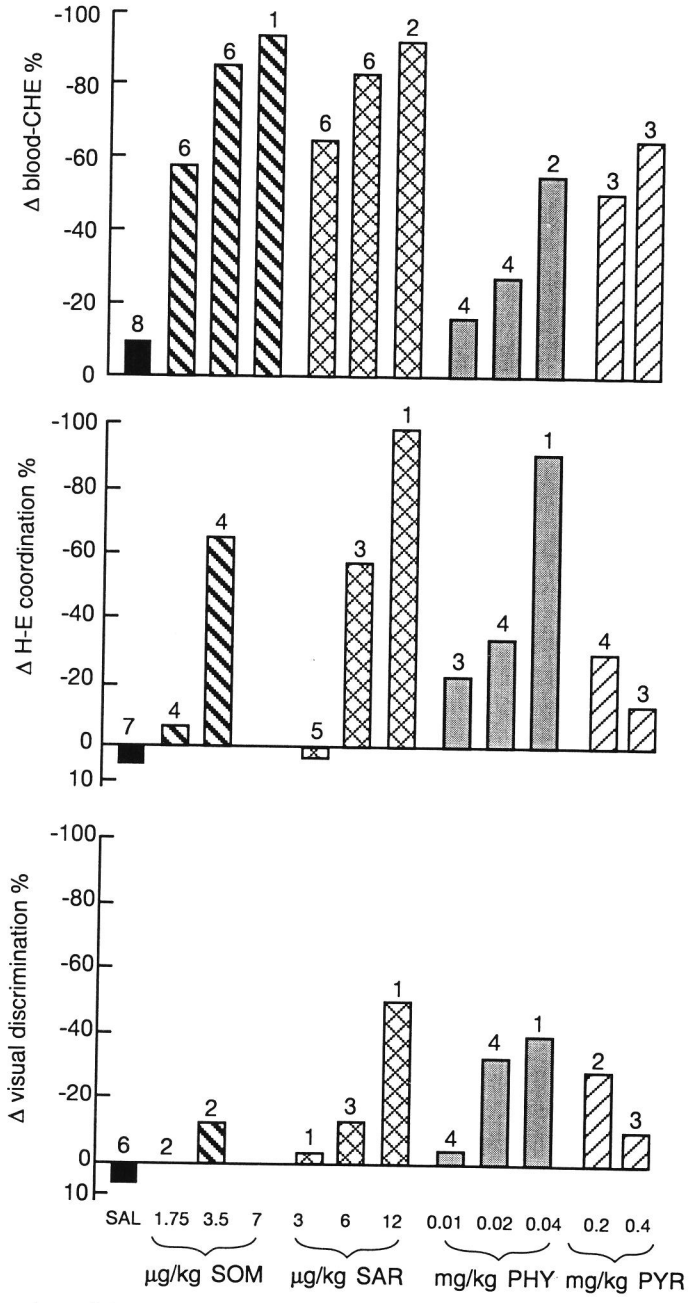


FIG.2. Overview of the effects of saline, soman, sarin, physostigmine, or pyridostigmine (doses at the botom) on the total blood ChE activity (top), on hand-eye (H-E) coordination (middle), and on visual discrimination performance (bottom). The numbers below the bars refer to the number of animals used. Mean ± SEM.

This work was supported by the U.S. Army Medical Research and Development Command under Grant DAMD17-88-Z-8020.

2.3

A simple automated test to measure exploratory and motor activity of marmosets

Otto L. Wolthuis, Bas Groen, Ingrid H.C.H.M. Philippens

TNO Medical Biological Laboratory (TNO-MBL), Research Group Pharmacology, Rijswijk ZH, The Netherlands.

An automated device is described to test the exploratory and motor activity of common marmosets *(Callithrix jacchus)*. The device consists of four boxes interconnected by PVC tubes. The presence of an animal in a box is detected by a photocell. Calibration takes place with an electric model train. Movements of the animal from one box to another are detected by disappearance from one and appearance in another box. The apparatus is linked to a PC. The effects of two doses of methamphetamine and of pentobarbital are shown.

Pharmacol Biochem Behav 47:879-881, 1994

Introduction

Although several automated devices have been developed to assess motor and exploratory activity in rodents (Sanberg *et al.*, 1985; Wolthuis and Vanwersch 1984), such devices are not available for marmosets.

Apart from observational (ethological) methods to count certain activities, to our knowledge only one type of locomotor activity monitoring has been applied to assess the activity levels of marmosets; D'Mello and Duffy (1985) used a device that was mounted on the back of an animal by a simple elastic harness. Movements in the anterorostral plane operated a simple mercury tilt switch; the counts were stored and could be read out.

The level of activity, alertness, and exploratory behaviour play an important role in practically all measurements of animal behaviour. Hence, we decided to develop a relatively simple device by which the combined locomotor and exploratory activity can be automatically and quantitatively assessed.

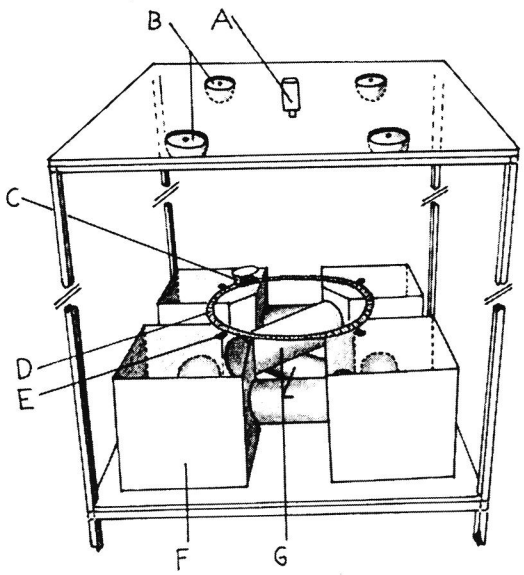


FIG. 1. A drawing of the bungalow test apparatus. (A) TV camera (B) lights; (C) locomotive with disk; (D) railroad; (E) photocell; (F) nontransparent box; (G) interconnecting PVC tubes. The whole apparatus is surrounded by a nontransparent curtain (not drawn).

Method

Animals

Experimentally naive marmosets (7 females and 10 males) with a body weight between 200-420 g were tested. Six animals were injected with saline, five animals with a low dose of the drugs, and six animals with a high dose of the drugs (see the Procedures section).

Apparatus

The apparatus (see Fig. I) consist of four horizontally placed non-transparent PVC boxes (25 x 25 x 25 cm) with a meshwire top, interconnected by PVC tubes (inner diameter 9.5 cm). It resembles a four-room bungalow. Hence, the test was called the bungalow test. The tubes are wide enough to allow the animal to move directly to each of the three other boxes. The boxes are placed in a square and the distance (heart to heart) of the boxes to the adjacent ones is 43 cm. Four lights are mounted on the closed ceiling of the apparatus, each one vertically 170 cm above the centre of the bottom of one of the four boxes. The floors of the boxes are made of white plastic and reflect the light. On each of the meshwire tops a photocell is mounted that is linked to an IBM-compatible PC.

A TV-camera is mounted in the centre of the ceiling to allow observation of the animal. The whole apparatus is surrounded by a thick curtain to avoid distraction of the animal.

Principle

The bottom of each box reflects the light that is registered by the photocells. The presence of a marmoset in the box is detected by the decrease in reflected light.

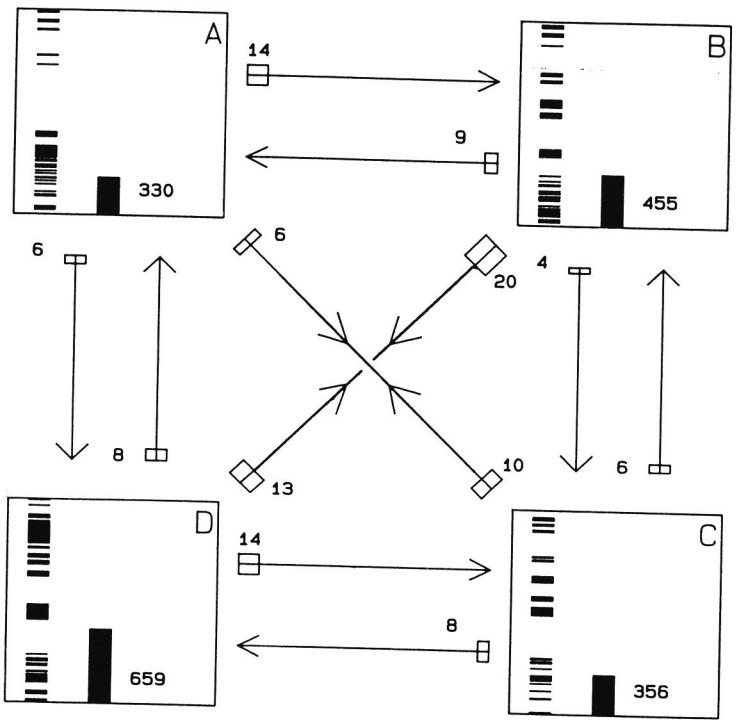


FIG. 2. An example of a computer printout of one marmoset in the bungalow test. The cumulative time in seconds that the animal spends in each box is indicated within each schematic representation of the box (see vertical bar and numbers). In addition, the various interrupted periods that the animal remains in a compartment are depicted. Moreover, the number of times that the animal leaves a compartment through one of the connecting tubes is also shown.

Registration

Software was developed that allows automated registration of: a) time spent and time intervals of the presence of the animals in each box, b) the number of times that the animal switches from one box to another, and c) from which box these switches take place. A schematic computer printout of the results from one animal, the arrangement of the boxes and interconnections, as well as the parameters measured, are shown in Fig. 2.

Calibration

This takes place with an electric Fleishman model minitrain (type Picolo, scale N). A locomotive, carrying a horizontal cardboard disk (diameter 8 cm) rides on a circle of rails, positioned in a standardised manner on the meshwire top of the boxes. Each time the locomotive rides over a box the light thrown into the box is slightly reduced and, consequently, reflection of light via the bottom is decreased, which is detected by the photocell. When calibrated during 20 min, an equal and reproducible number of switches and periods of time spent in each box should be registered.

Experimental Procedures

Testing lasted 20 min. Recordings were taken for each animal and averaged per group. Control measurements were performed twice; the results of the second control test were taken as the starting value for each animal. The day after the second test the animals were injected intramuscularly with saline (n=6) or methamphetamine in doses of 0.5 mg/kg (n=5) or 1.0 mg/kg (n=6). Testing of these animals started 30 min after the injection. Subsequently, a number of animals in the drug-treated groups were interchanged, in such a way that a number of animals that had received a low dose of drug would subsequently receive a high dose of the next drug and vice versa.

Two weeks after methamphetamine had been given, again, a control test was carried out to have a starting control value for the next drug to be tested. The day after this control test the group that had previously received saline received saline again (n=6) and the other two groups received an i.m. injection of pentobarbital in doses of 4.0 mg/kg (n=5) or 8.0 mg/kg (n=6), respectively. Thirty minutes after injection testing started.

Statistics

For statistical comparisons, the multiple t-test of Welch (Natrella *et al.*, 1963) was applied. When the term significant is used, this indicates a $p < 0.05$ (two-tailed).

Results

The averaged time spent in each of the four boxes did not differ significantly. Before any injection was given, the largest difference in the time spent in the second control test at the beginning of the experiment was 268 $\pm$ 30.1 s in box A (see computer printout) and 349 $\pm$ 34.0 in box C ($p_2 = 0.084$). All 17 animals visited all four boxes. Similarly, no significant differences were found with respect to the use of the connecting tubes. Here the largest difference was found between the use of the tubes going from box B to D (mean $\pm$ SEM: 2.2 $\pm$ 0.58 times and that of D to C 3.4 $\pm$ 0.59 times). Both tubes were not used by 3 out of the 17 animals.

An interesting feature of this technique for marmosets is, that preliminary experiments showed, when a separate group of six saline-injected animals was tested four times in 1 week during 30 min, that the averaged number of compartment changes of the group to each box remained stable and even showed a slight and insignificant increase:

	Number of compartment changes				
Date:	Sept 5	Sept 6	Sept 7	Sept 12	n
Mean (± SEM)	108 (26)	131 (23)	141 (35)	132 (25)	6

The effects of the two drugs tested (see Fig. 3: compartment changes) were as expected; both doses of methamphetamine caused a significant increase in number of visits to each box whereas this number decreased significantly following injection of 8 mg/kg, but not significantly after 4 mg/kg pentobarbital.

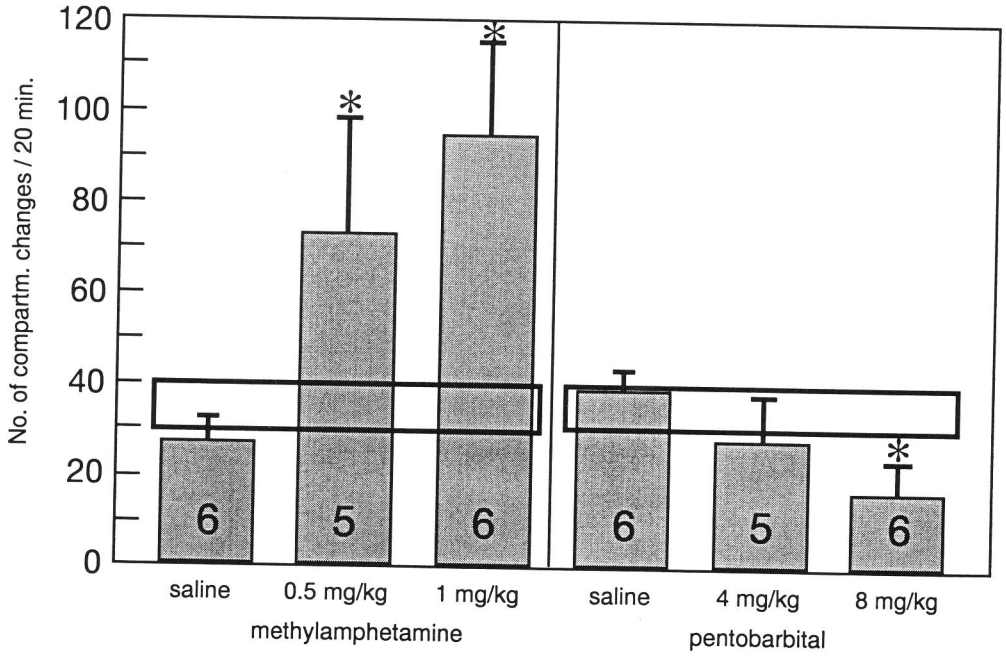


FIG. 3. The effects of intramuscular methamphetamine and pentobarbital on the number of compartment changes. The tests with pentobarbital were conducted with the same animals, after a rest period of 14 days following the injections of methamphetamine. The shaded horizontal bar represents the mean (± SEM) control number of compartment changes tested on the day before the injections with each drug took place. *Significantly different from effect of saline.

Discussion

The bungalow test presented offers the possibility to quantify locomotor and exploratory behaviour of marmosets in a simple and automated fashion and can -in principle- also be used for other small animal species. The tests conducted so far indicate that the method is fairly robust. A pleasant surprise was that the exploratory activity did not drastically reduce upon repeated testing, resulting in a rather stable base line, at least on four successive tests during one week. The number of compartment changes of these six animals is much larger than that of the animals used for testing the two drugs, even when it is taken into account that these six animals were tested during 30 min and the drug-treated animals only during 20 min. This was not due to a sudden increase in activity in the additional 10 min, but most likely due to the fact that these six animals had been in the animal room for at least a year and were used to being handled and being exposed to several test situations. The 17 animals used to test the drugs were new animals, had been in the animal room during only 2-3 weeks, and had never been injected before. Thus, it seems likely that the six animals used for repeated testing had a reduced level of fear for novel situations.

The four horizontally placed identical boxes seem equally attractive to the animals, because the time they spend in each of the four boxes is roughly the same. A restriction of the method is that vertical mobility components of these behaviours are not measured; this would require a more elaborate apparatus.

Although the tests are different, a comparison with the effects of d-amphetamine and pentobarbital on a visuo-motor coordination task of D'Mello *et al.* (1985) suggests that the present test is more sensitive to the activity-enhancing effect of an amphetamine-like drug, but slightly less sensitive to an activity-reducing drug like pentobarbital. The latter authors found no changes in performance with i.m. doses up to 2 mg/kg *d*-amphetamine, but did find a small significant depression after an i.m. dose of 4 mg/kg pentobarbital. In principle, this test offers the possibility to test antianxiety drugs. This could be achieved by introducing in one of the boxes an aversive stimulus (e.g., a photograph of a male marmoset in an aggressive posture, or of an unknown human observer (Carey *et al.*, 1992)). By subsequently measuring whether a) marmosets would avoid that box, and b) whether an anxiolytic would counteract that effect, the efficacy of an anxiolytic could be assessed.

The results of preliminary tests, however, suggest that marmosets are not easily intimidated by either pictures or by video films of snakes, tigers, sounds of exploding in war movies, or movies with hard rock music and flashing lights. The animals were exposed to these pictures and movies by making a wall of a box transparent with Plexiglas and by placing a TV monitor directly in front of this transparent wall. In contrast, the animals seemed quite interested in what was shown. We will continue the search for an effective stimulus that evokes anxiety.

3

Side effects of physostigmine as a pretreatment in guinea pigs

Ingrid H.C.H.M. Philippens, Otto L. Wolthuis, Ruud W. Busker, Jan P. Langenberg and Bert P.C. Melchers

TNO Prins Maurits Laboratorium (TNO-PML)
Researche group Pharmacology

To prevent incapacitation following nerve agent intoxications, it is proposed to replace pyridostigmine by the centrally active carbamate physostigmine (PHY). Behavioural 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-nitrophenyl phosphoramidate (PNF). At similar levels of blood AChE-inhibition, PHY caused a larger shuttlebox performance decrement than PNF, which was antagonised 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 antagonised 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 which are unrelated to AChE-inhibition.

Pharmacol. Biochem. Behav. 55:99-105, 1996

Introduction

The current pretreatment/treatment regime against intoxication with nerve agents, i.e. 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 (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978; Harris *et al.*, 1980). 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 behavioural incapacitation that usually follows OP-intoxication.

Leadbeater *et al.*(1985) 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.* (1985) 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 behavioural 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 (Langenberg *et al.*, 1989).

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 e.g. on nicotinic ACh receptors (Albuquerque *et al.*, 1984; Bakry *et al.*, 1988; Sherby *et al.*, 1984).

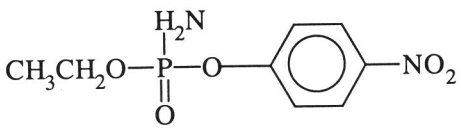


FIG. 1: Ethyl p-nitrophenyl phosphoramidate (PNF)

Material and 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-nitrophenyl phosphoramidate (PNF) was synthesised by dr. H.P. Benschop PML TNO, Rijswijk (.Langenberg *et al.*, 1988). Eserine (Physostigmine) and scopolamine bromide were obtained from Sigma Chemical Co., St.Louis, USA.

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 5-8 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 min. 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 24h later.

1) Shuttlebox performance

Shuttlebox performance was determined as described earlier (Philippens *et al.*, 1992a). In short an automated two-way shuttlebox was used, consisting of two equal compartments of 23x23x23 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).

2) *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.

3) *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 anaesthesia. 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 immobilised in a vertically mounted PVC tube (as for the startle response). Fourier transformation (FFT), to obtain power spectra, was performed on line from 5 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 (50.000x), 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.

4) *Determination of AChE-activity*
Blood samples (5µl) 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 (Johnson and Russell, 1975). The ACh end-concentration used was 12 µm;[^{3}H]ACh iodide (NEN, Dreicich, Germany) was diluted to a specific activity of 602 MBq.mmol^{-1}. Ethopropazine (2.5 µM, St. Louis, Mo, USA) was used as a specific inhibitor of butyrylcholinesterase. Electric eel AChE was used as a reference.

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.

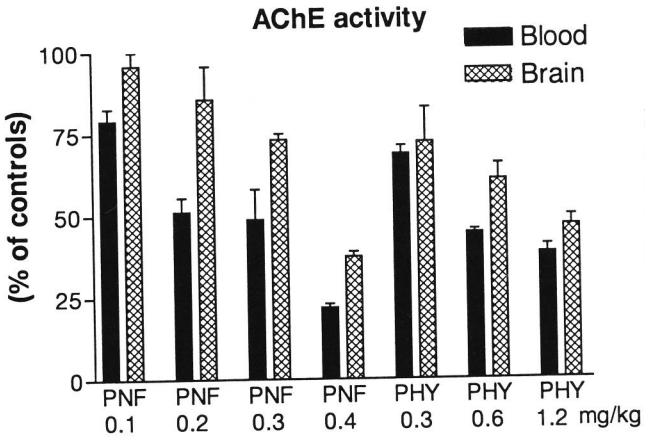


FIG.2:The AChE-activity in blood and brains of guinea pigs, 30 minutes after the s.c. injections of PNF (0.1, 0.2, 0.3 or 0.4 mg/kg) or physostigmine (Phys, 0.3, 0.6 or 1.2 mg/kg).

Results

The sc doses of the AChE inhibitors, necessary to obtain therapeutically relevant levels of AChE-inhibition in blood (Leadbeater *et al.*, 1985) 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, i.e. 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 resp. 28% and 62%, which is more comparable with the brain AChE-inhibition obtained with PHY 0.6 mg/kg.

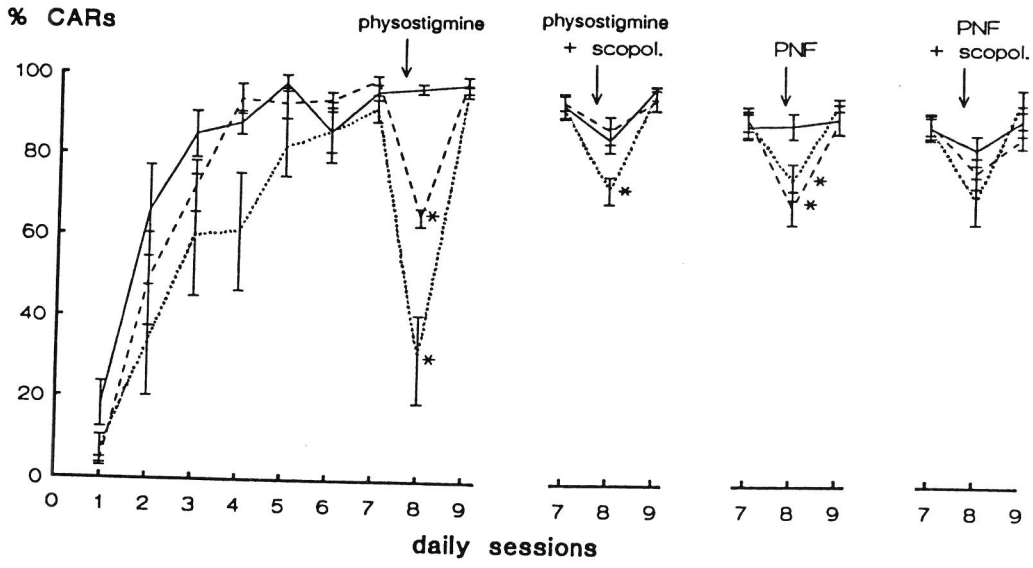


FIG. 3: Mean (± SEM) performance of guinea pigs in the shuttlebox task of four experiments. Animals were trained (CS, sound; UCS, air stream; 1 session/day, 20 trials/session) until >80% CARs were reached, usually on day 7. On day 8, the drugs were injected and 30 min later another session started. Animals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

In the shuttlebox task the acquisition rates for the four groups obtained in the experiments described in this paper 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 [$F(2,20)=6,54$, $p=0.007$] 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 antagonised 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.

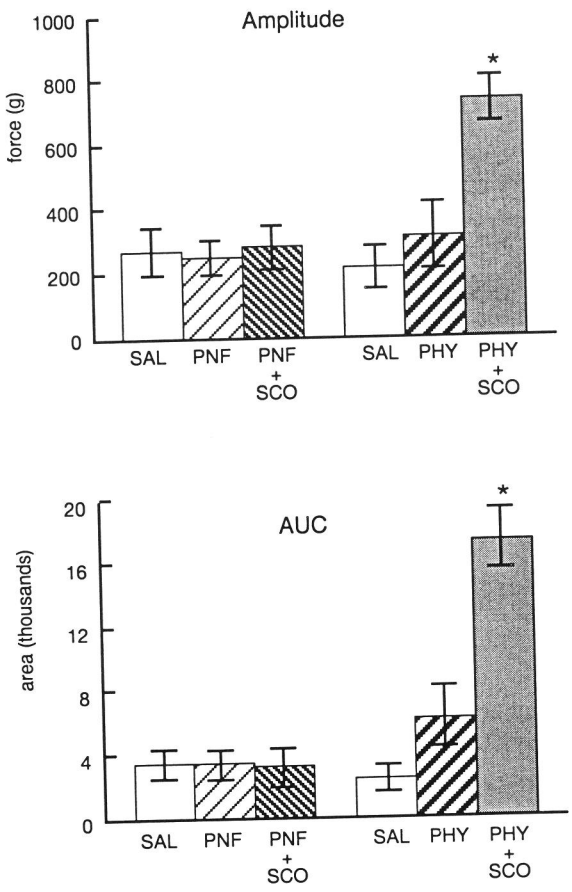


FIG. 4: Mean (± SEM) of the amplitude and AUC of the startle response (startle pulse: 120 db, 10 Hz, 20 ms). Registration of the effects 30 min after sc injection of: saline or PNF (0.4 mg/kg) or PNF (0.4 mg/kg) + SCO (0.1 mg/kg), n=8 animals/group and saline or PHY (physostigmine)(0.6 mg/kg) or PHY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

At a dose of 0.6 mg/kg, PHY caused a small increase of the startle response that 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 hour later the responses were on pre-injection level. PNF, at a dose of 0.2 mg/kg, 0.3 mg/kg nor at a dose of 0.4 mg/kg (Fig. 4), had any effect on the startle response. The combination of scopolamine and PNF was also without effect [ampl.: $F(2,15)=0.63$,$p=0.547$ and AUC: $F(2,15)=0.06$, $p=0.937$]. Scopolamine in doses of 0.0 or 0.1 or 0.2 or 0.4 mg/kg had no effect [$F(3,20)=0.88$, $p=0.467$] on the startle response amplitude (resp. 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 antagonised 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 antagonised 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.

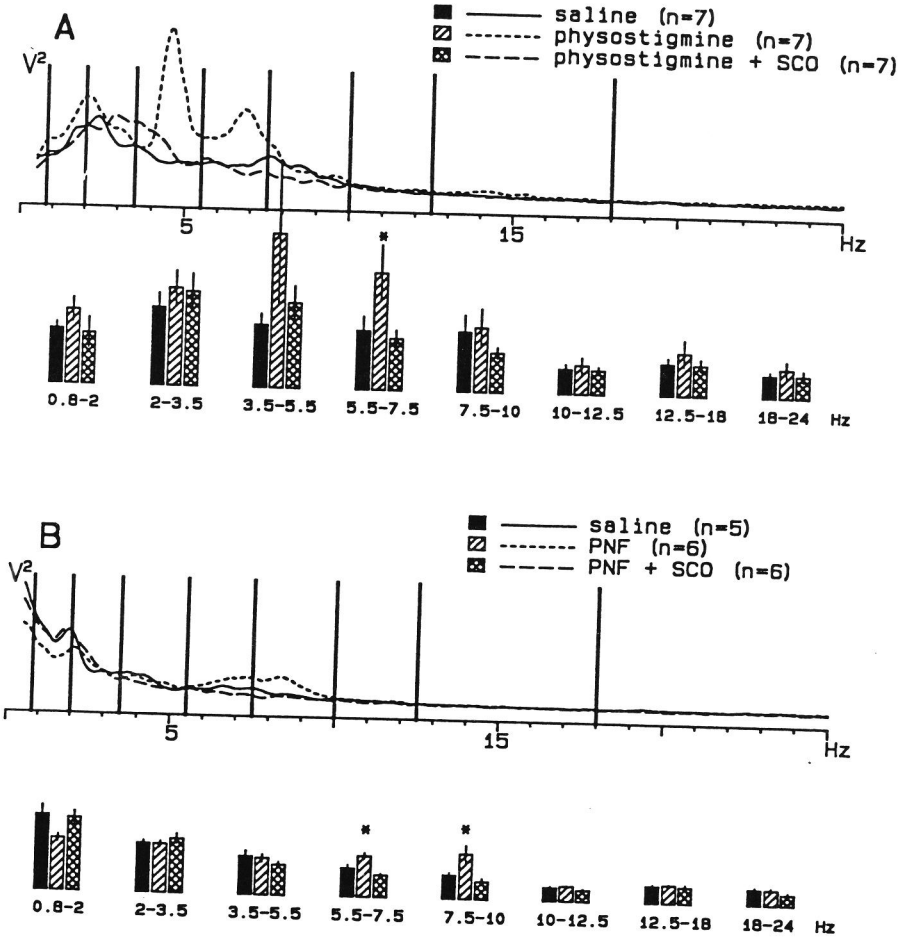


FIG. 5: Power spectra of EEGs obtained from guinea pigs. The vertical lines divide the frequency classes, the power of which is represented by bars at the bottom. Absolute power is shown (V^2). Registration of the effects 45 min after sc injection of: A: saline, physostigmine (0.6 mg/kg), or physostigmine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

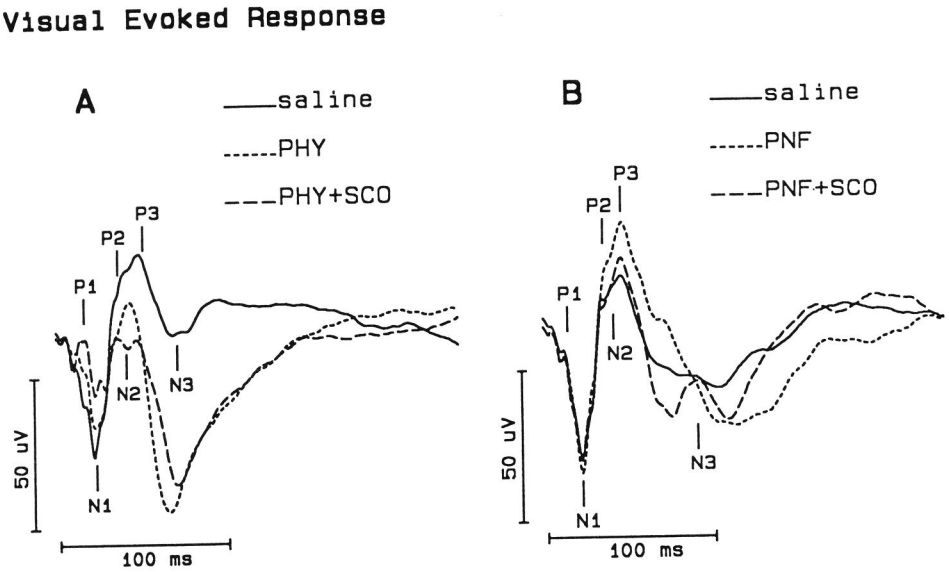


FIG. 6:
The averaged visual evoked response (VER) curves from guinea pigs (N=8 animals/group). Registration is shown 45 min after sc injection of: A: saline, physost.(physostigmine) (0.6 mg/kg), or physost. (0.6 mg/kg) + SCO (scopolamine) (0.1mg/kg) and B: saline, PNF (0.2 mg/kg), or PNF (0.2 mg/kg) + 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, 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.

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 [$F(2,21)=4.49$, $p=0.024$]; N2: PHY/SCO: -18 ± 10.0, saline: 29 ± 12.2 [$F(2,21)=4.71$, $p=0.02$]; P3: PHY/SCO: 5 ± 7.4, saline: 54 ± 10.4 [$F(2,21)=6.76$, $p=0.005$]; 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) [$F(2,14)=4.80$, $p=0.026$].

Discussion

In this study we used different neurophysiological and behavioural 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 (Davis *et al.*, 1982). Muscarinic receptors appear not to be involved in the increased startle response. Since scopolamine is a competitive antagonist of ACh at the muscarinic receptor and since ACh-accumulation also occurs at the nicotinic synapses, a complex picture emerges. Scopolamine may increase ACh-release (Consolo *et al.*, 1991). That nicotinic receptors may be involved in the startle response may be inferred from the results of Acri *et al.*(1991), 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 e.g. of nicotinic ACh receptors (Albuquerque *et al.*, 1984; Bakry *et al.*, 1988; Sherby *et al.*, 1984). Whatever the explanation, the different effects caused by PHY and PNF, whether or not in combination with scopolamine, do indicate that more processes are involved than AChE-inhibition alone.

On shuttlebox performance, a subcutaneous 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 which 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. Since scopolamine effectively antagonised 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 (Tomas and Gralewicz, 1992). This is consistent with the results of our earlier studies, in which we tested a therapy against intoxication 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 which is indicative for neuropathology. After therapy with higher doses of these drugs, offering a better protection against convulsions and lethality, an increase of the alpha1 power (7.5-9.9 Hz) was found which might reflect increased cortical cholinergic stimulation due to persisting cholinesterase inhibition (Philippens *et al.*, 1992b). 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 cause several undesirable side effects which 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 of 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 (Chapter 5) show that upon subchronic administration these symptoms were not found.

4

Effects of physostigmine on the startle in guinea pigs: Two mechanisms involved

Ingrid H.C.H.M. Philippens[1], Berend Olivier[2] and Bert P.C. Melchers[1]

[1]*Research group Pharmacology,TNO Prins Maurits Laboratory (TNO-PML), Rijswijk, The Netherlands.*

[2]*Dept. of Psychopharmacology, Rudolf Magnus Institute for Neurosciences, Fac. of Pharmacy, Utrecht University, Utrecht, The Netherlands.*

The effects of the acetylcholinesterase inhibitor physostigmine (PHY) on the auditory startle reflex in guinea pigs were studied. The dose response curve of PHY appeared bell-shaped, with a maximum effect dose of 0.3 mg/kg. In addition, PHY altered the shape of the startle response. The muscarinic antagonist scopolamine (SCO) increased the startle at PHY doses above 0.3 mg/kg without affecting the PHY-induced shape of the response. The decreasing part of the startle due to PHY could be mimicked by the cholinesterase inhibitor soman in combination with 0.3 mg/kg PHY. It appeared that the decreasing part of the dose response curve at higher dose levels is caused by the cholinesterase inhibitory action of PHY and, in view of the SCO effect, is mediated by muscarinergic receptors. The increasing part of the curve is probably caused by an agonistic action of PHY on neuronal nicotinergic receptors, since the antagonist mecamylamine (20 mg/kg) antagonised the effects of 0.3 mg/kg PHY both on the deflection and shape of the startle.

Pharmacol. Biochem. Behav. 58:909-913, 1997

Introduction

The acoustic startle response in rodents is a sensitive method to determine how different neurotransmitter systems modulate sensorimotor activity (Davis, 1980). The role of the cholinergic system in modulating the startle reflex is far from clear. No consistent effects of cholinergic drugs on the startle response have been reported (Hughes, 1984; Wu *et al.*, 1993; Overstreet, 1977; Handley and Thomas, 1979). These studies therefore support the conclusion of Davis (1980) that the cholinergic system only plays a small and indirect role in the startle response. However, in a study (Philippens *et al.*, 1996) on behavioural side effects of the combination of the acetylcholinesterase inhibitor physostigmine (PHY) and the muscarinic antagonist scopolamine (SCO), we noticed unexpected effects on the startle reflex. While some behavioural and neurophysiological side effects, caused by PHY, could be antagonised by a low dose of SCO, the addition of SCO considerably enhanced, and not antagonised, the increase of the startle response. It is unlikely that the effects of PHY on the startle response are caused by its acetylcholinesterase (AChE)-inhibiting effect because these effects have not been reported for other AChE-inhibitors. Diisopropyl fluorophosphate slightly enhanced the startle reflex (Davis, 1980) while another organophosphate AChE inhibitor, soman, slightly decreased it (unpublished data). Para-nitrophenyl phosphoramidate, a reversible organophosphate AChE-inhibitor (Langenberg *et al.*, 1988), alone or in combination with SCO had no effect on the startle although AChE-inhibition in the brain was similar as found after PHY-administration (Philippens *et al.*, 1996). This indicates that besides AChE-inhibition additional effects of PHY may be involved in its effects on the startle response.

Such effects of PHY, unrelated to AChE-inhibition, have been reported earlier (Albuquerque *et al.*, 1984; Bakry *et al.*, 1988; Sherby *et al.*, 1984). PHY may have both agonistic and antagonistic effects on nicotinergic ACh receptors (Albuquerque *et al.*, 1984; Bakry *et al.*, 1988; Sherby *et al.*, 1984). In the present study an explanation is given for the previous reported effects of PHY, SCO and their combination (Philippens *et al.*, 1996) on the startle reflex.

Material and Method

Animals

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

General procedure

In order to obtain control values, the startle response was measured in all animals one day before drug injections. Subsequently, on the basis of the obtained results, comparable subgroups were formed of 5 or 6 animals each. Thereafter, the animals were subcutaneously injected with the drugs under investigation. Startle responses were measured 30 minutes and 24 hours after injection. Only those animals with pre-drug startle responses of more than 100 g were used in analysing of the drug effects.

Auditory startle response

The animals were exposed to 20 auditory startle pulses while standing in a vertically mounted PVC-tube (diameter 7 cm, length 16.5 cm), resting with their hind paws on a platform. The startle-eliciting stimulus consisted of a 20 ms, 120 dB, 10 kHz band-pass filtered burst of white noise. Startle responses were measured by a transducer connected with the platform. For the duration of 100 ms and in later experiments 200 ms the force exerted by the hind paws upon presentation of the stimulus was registered. In this way only the startle response of the hind paws was recorded. The data were digitised (50 Hz) by the ADC of an IBM compatible personal computer, averaged and stored on disk for later analysis. The area under the curve (AUC) measured for the duration of 100 ms after presentation of the startle pulse was used to quantitate the startle reflex. Only in the last experiment we measured the AUC during 200 ms.

Statistics

An analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test was used to assess statistical significance. In case pre- and post drug values were compared in one animal the Wilcoxon matched pairs signed rank test was used. P values < 0.05 were considered significant.

Drugs

Physostigmine (eserine) and scopolamine bromide were obtained from Sigma, St.Louis, U.S.A.; Mecamylamine hydrochloride was obtained from Merck Sharp & Dohme International, Pahway, U.S.A.; Soman (O-pinacolyl methylphosphonofluoridate) was synthesised at the Prins Maurits Laboratory TNO (dr H.P.Benschop).

Results

The PHY dose-response curve (DRC) on the startle reflex is shown in Fig.1. It shows a bellshaped response curve; the maximal effective dose of PHY being around 0.3 mg/kg [$F(4,18)=4.67$; $p=0.009$]. The effect of PHY is not only an effect on the amplitude of the startle. At all doses used, PHY induces a change of the shape of the startle response (Fig. 2). After having reached the maximal amplitude, the curve did not return to baseline within the 100 ms registration time, but showed a "shoulder". When longer registration periods (200 ms) were applied it lasted about 140 msec before the startle response returned to baseline (Fig. 6).

SCO (0.1 mg/kg) combined with different doses of PHY (Fig. 1) only had an increasing effect at doses of PHY higher than the maximal effective dose, but did not affect the "shoulder" in the response curve [$F(1,8)=7.16$; $p=0.028$]. The percentual changes induced by SCO (0.1 mg/kg) at the two higher PHY dose levels, as compared to PHY were significantly increased [$F(3,18)=5.66$; $p=0.007$]. This curve also appeared to be bellshaped.

The decrease of the startle reflex at the highest dose of PHY compared with the combination of SCO with PHY (0.6 mg/kg) was not caused by an incomplete antagonistic activity of SCO. The SCO dose tested (0.1 mg/kg) appeared to be already maximally effective; higher and lower doses of SCO did not result in a significantly larger or smaller enhancement of the startle (Fig. 3) [$F(4,22)=0.64$; $p=0.64$].

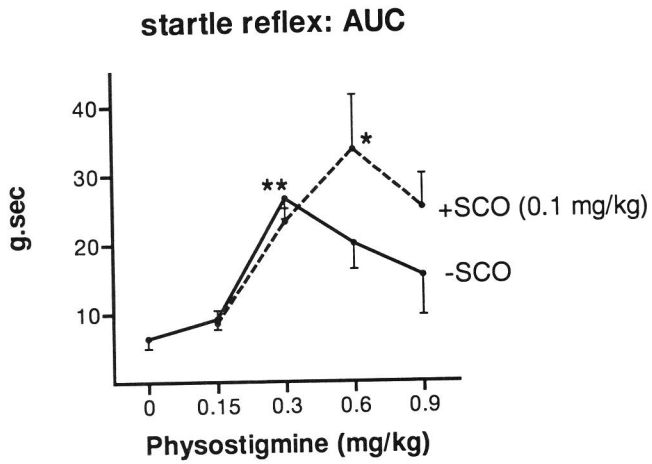


FIG. 1. Mean (± SEM) of the AUC of the startle response of 100 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects 30 min after subcutaneous injection of: saline, PHY (0.15, 0.3, 0.6, or 0.9 mg/kg), or PHY (0.15, 0.3, 0.6, or 0.9 mg/kg) + SCO (0.1mg/kg), n=5 or 6 animals/group

*Significantly different, between PHY and PHY/SCO treated groups, using analysis of variance and Newman-Keuls post-hoc test $p<0.05$.

**Significantly different, between PHY and control value, using analysis of variance and Newman-Keuls post-hoc test $p<0.05$.

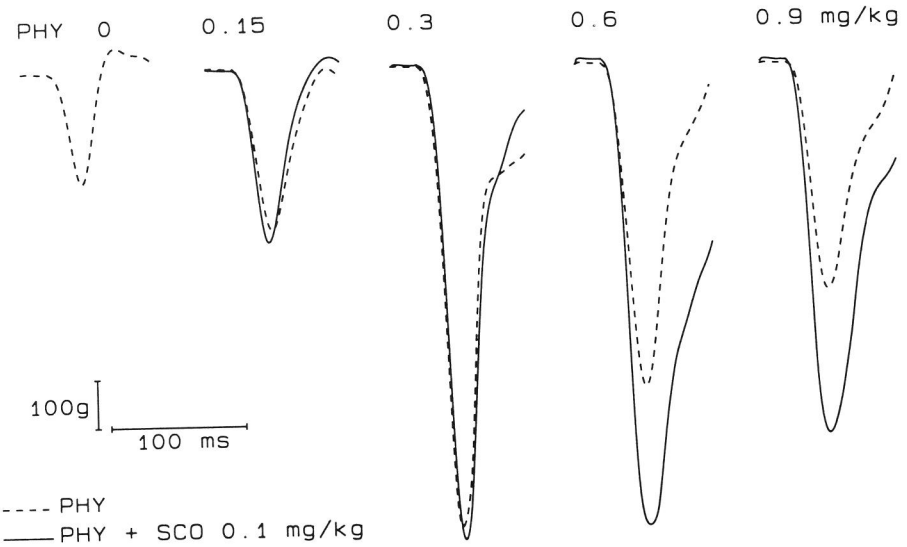


FIG. 2. Mean of the startle response of 100 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects 30 min after subcutaneous injection of: PHY (0, 0.15, 0.3, 0.6, or 0.9 mg/kg): dotted lines, or PHY (0.15, 0.3, 0.6, or 0.9 mg/kg) + SCO (0.1mg/kg): bold lines, n=5 or 6 animals/group.

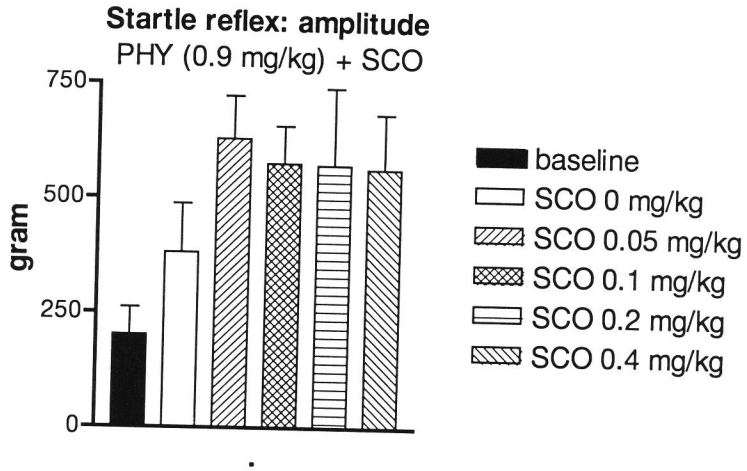


FIG. 3. Mean (± SEM) of the amplitude of the startle response of 100 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects 30 min after subcutaneous injection of saline, PHY (0.9 mg/kg) + SCO (0.05, 0.1, 0.2, or 0.4 mg/kg), n=6 animals/group.

These drugs didn't affect general motor activity: the number of intertrial responses, a measurement of the activity level, obtained in an active avoidance task, showed no differences between before and after injection (SCO 0.1 mg/kg [F(1,12)=1.87, p=0.196]; PHY 0.6 mg/kg [F(1,14)=2.18, p=0.162] or 1.2 mg/kg [F(1,14)=0.49, p=0.494]) (13).

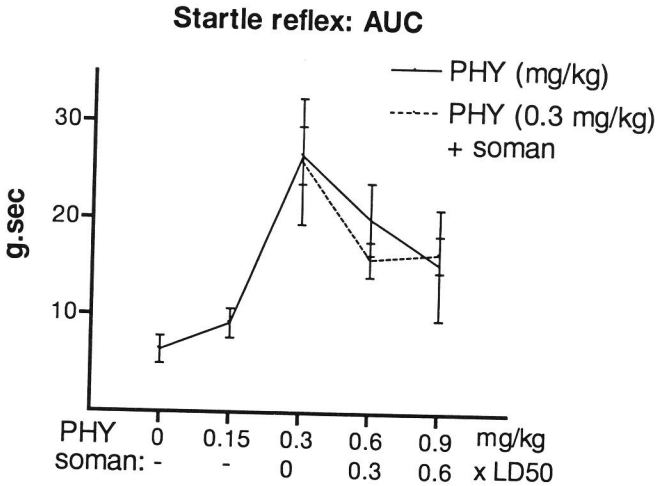


FIG. 4. Mean (± SEM) of the AUC of the startle response of 100 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects 30 min after subcutaneous injection of PHY (0.3 mg/kg) + Soman (0xLD_{50} or 0.3xLD_{50} or 0.6xLD_{50}), n=6 animals/group, compaired with the DRC of PHY. The LD_{50} dose of soman is 0.025 mg/kg sc (Gordon and Leadbeater, 1977).

To find out whether the effect of PHY at higher dose levels was caused by the AChE inhibitory capacity of PHY, we tried to mimick the descending second phase of the DRC of PHY by applying low doses of the AChE-inhibitor soman combined with 0.3 mg/kg PHY. In Fig. 4 the DRC of PHY is compared with the curve composed of the maximal effect dose of PHY and PHY in combination with two different dosages of soman (0.3 and 0.6 x LD_{50} (=0.025 mg/kg)(Gordon and Leadbeater, 1977). These curves appeared similar [F(2,15)=1.96; p=0.17]. However, soman did not affect the shape of the startle response after PHY. A single administration of soman at the highest dose (0.015 mg/kg sc) resulted in a small but insignificant decrease of the startle response compared to a control group before and 30 minutes after injection [F(3,44)=2.04, p=0.122].

To investigate the involvement of nicotinergic receptors, the effect of the neuronal nicotinergic receptor antagonist mecamylamine (MMA) was tested in combination with the maximal effect dose of PHY. MMA at a dose of 20 mg/kg sc caused a small but significant increase of the startle compared with the pre-injection value (P=0.05, Wilcoxon matched pairs signed rank test). MMA (20 mg/kg) completely antagonised the effect of PHY (0.3 mg/kg): the startle response after the combination of MMA and PHY was significantly smaller than the startle found after PHY (0.3 mg/kg) alone (Fig. 5) [F(2,15)=5.41; p=0.017]. Furthermore, the typical "shoulder" in the startle response always present after PHY administration, also disappeared when PHY was given in combination with MMA (Fig. 6).

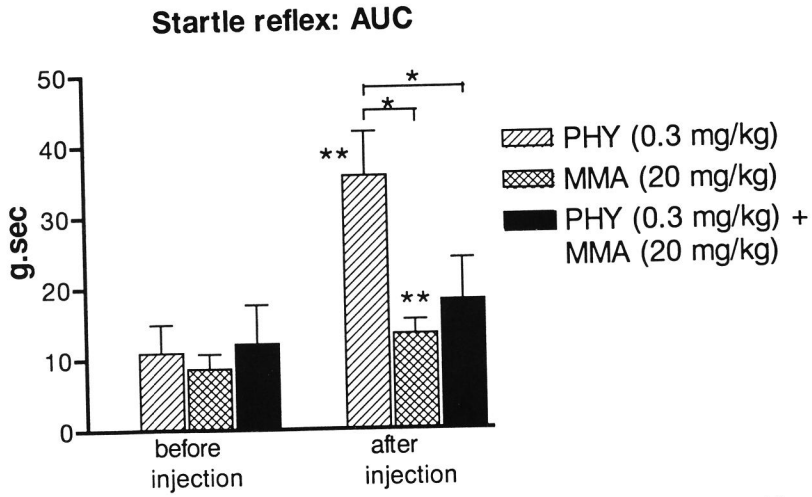


FIG. 5. Mean (± SEM) of the AUC of the startle response of 200 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects 30 min after subcutaneous injection of Mecamylamine (MMA) (20 mg/kg), PHY (0.3 mg/kg), or MMA (20 mg/kg) + PHY (0.3 mg/kg), n=6 animals/group.
*Significantly different using analysis of variance and Newman-Keuls post-hoc test $p<0.05$.
**Significantly different using Wilcoxon matched pairs signed ranked test $p<0.05$.

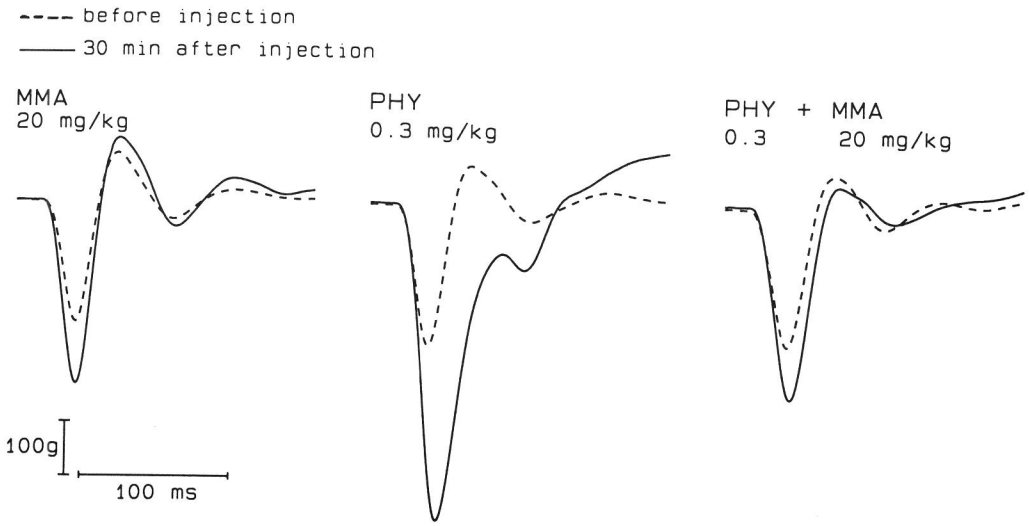


FIG. 6. Mean of the startle response of 200 ms duration (startle pulse: 20 ms, 120 dB, 10 kHz). Registration of the effects after subcutaneous injections of Mecamylamine (MMA) (20 mg/kg), PHY (0.3 mg/kg), or MMA (20 mg/kg) + PHY (0.3 mg/kg), before injection: dotted lines, or 30 minutes after injection: bold lines, n= 6 animals/group.

Discussion

In this study the pharmacology of the effects of PHY on the auditory startle reflex of the guinea pig were investigated. According to Davis (1980) ACh only plays a minor or indirect role in the startle modulation. This view seems justified when the effects on the startle response of different types of cholinesterase inhibitors are considered: only small effects were reported (Davis, 1980; Philippens *et al.*, 1996). However, our present data disagree with this opinion. We demonstrated a clear enhancing effect of PHY on the startle response, especially when PHY was combined with SCO. This can not be due to general motor effects since previously it was demonstrated (see results) that the motor activity was not influenced.

As argued before, it is unlikely that this enhancing effect of PHY can be ascribed to inhibition of AChE, because other cholinesterase inhibitors have failed to induce such an effect, even at doses leading to larger levels of brain AChE inhibition (Philippens *et al.*, 1996). Therefore, additional effects of PHY may be involved. Several effects of PHY, other than inhibition of AChE have been described. PHY may act both as an agonist and an antagonist on nicotinergic receptors (Albuquerque *et al.*, 1984; Bakry *et al.*, 1988; Sherby *et al.*, 1984). Interestingly, the ED_{50} of PHY's agonism at the nicotinergic receptor appears to be lower than its IC_{50} of AChE inhibition (Albuquerque *et al.*, 1988). In view of the antagonism of MMA, a neuronal nicotinergic receptor antagonist, on the PHY effects on the startle, our results may be explained by an agonistic action of PHY on these receptors. This is in agreement with the results of Acri *et al.*(1991), who have

shown that nicotine causes a dose-dependent increase of the startle response. This effect of PHY apparently occurs already at very low PHY concentrations in the brain. A change of the shape of the startle response, showing a characteristic shoulder, was seen even at the lowest PHY dose we used (0.15 mg/kg)(Fig.2). The slight increase of the startle found after MMA might be due to stress factors caused by the injection.

Interestingly, it appeared that when the AChE-inhibition in the brain reaches a certain level, the extra stimulation of cholinergic receptors leads to a decrease of the startle response that was increased by low doses of PHY. Therefore, it appears that PHY antagonises its own effect on the startle response. This latter effect of PHY can be mimicked by giving another AChE-inhibitor soman and may be antagonised by the muscarinic antagonist SCO. SCO at doses of 0.1, 0.2 or 0.4 mg/kg had no effect on the startle response (Philippens *et al.*, 1996). Activation of this inhibitory cholinergic system leads to a decrease of the startle. However, in view of the lack of effect of other AChE-inhibitors on the startle (Davis, 1980; Philippens *et al.*, 1996), this system is only effective when the startle is increased following PHY administration.
This is corroborated by others: PHY, at higher dose levels (i.e. via AChE-inhibition), activates neurons inhibiting the primary startle pathway. It appears that the pedunculopontine tegmental nucleus (PPTg) plays an important role in modulating sensorimotor gating by linking the ventral pallidum and the nucleus reticularis pontis caudalis, an obligatory part of the primary startle (Davis *et al.*, 1982; Swerdlow *et al.*, 1992; Swerdlow and Geyer, 1993), via a direct, presumably muscarinic, cholinergic projection (Koch *et al.*, 1993). This inhibitory circuit can be activated by acetylcholine agonists (Koch *et al.*, 1993). Furthermore, lesions of the PPTg lead to an increased startle amplitude (Swerdlow and Geyer, 1993).

However, not all effects of PHY appear to be antagonised by AChE-inhibition, the characteristic shoulder in the startle response remains present at all dose levels of PHY tested. This shoulder is responsible for the fact that the startle response curve found after PHY does not reach the baseline within 100 ms. Normally the response curve reaches the baseline within 100 ms which is seen in the control responses. This could lead to an underestimation of the effects established.
The nicotinergic receptor antagonist MMA was the only drug in this experiment that could also antagonize the shape of the startle curve.
On the basis of our results it is not possible to decide whether the nicotinic actions of PHY directly affect the startle or that other transmitter systems are involved. It has, for example, been shown that nicotine may enhance the release of 5-HT (Ribeiro *et al.*, 1993), and it has been shown that one of the major transmitter systems involved in the startle reflex is the serotonergic system (Davis, 1980).

In conclusion, the results demonstrate that PHY affects the startle response by two different but coupled mechanisms. A startle activating mechanism which is most likely due to an agonistic action of PHY on nicotinergic receptors and another a startle inhibiting mechanism which is most likely due to activation of muscarinergic receptors which are triggered after activation of the nicotinergic system.

5

Subchronic physostigmine pretreatment in guinea pigs: effective against soman and without side effect

Ingrid H.C.H.M. Philippens[1], Ruud W. Busker[1], Otto L. Wolthuis[1], Berend Olivier[2], Piet L.B. Bruijnzeel[1], Bert P.C. Melchers[1],

[1]*TNO Prins Maurits Lab (TNO-PML), Research Group Pharmacology, Rijswijk ZH, The Netherlands.*

[2] *Dept. of Psychopharmacology, Rudolf Magnus Institute for Neurosciences, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands.*

The behavioural and neurophysiological effects of the subchronically administered cholinesterase-inhibitor physostigmine (PHY) (0.025 mg/kg/hr) either with or without the muscarinergic antagonist scopolamine (SCO) (0.018 mg/kg/hr) were determined in guinea pigs. In contrast to a single injection of PHY, subchronic application by osmotic mini-pumps of PHY, even without SCO, caused no behavioural 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 x LD_{50} dose of soman.

Pharmacol. Biochem. Behav. 59:1061-1067, 1997

Introduction

Exposure to nerve agents is currently not restricted to the battlefield. 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. Since 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 (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978; Harris *et al.*, 1980). 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 (Leadbeater *et al.*, 1985). 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 (Leadbeater *et al.*, 1985). 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 behavioural and neurophysiological side effects (Philippens *et al.*, 1996). Part of these undesirable effects appears to be caused by AChE inhibition in the CNS and are counteracted by scopolamine (SCO) (Leadbeater *et al.*, 1985; Philippens *et al.*, 1996). However, some effects of 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.

Since 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 mini-pumps 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 mini-pump). This level of blood AChE inhibition exceeds that of the recommended prophylactic level of AChE inhibition in case of PYR (Gall, 1981). However, a 'worst' case approach seems to be appropriate when studying side effects of a pretreatment compound. Behavioural 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 mini-pumps

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

Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.5 µl/hr (Model 2002, Alza Corp., Palo Alto, CA) were used to deliver either the vehicle, PHY (0.025 mg/kg/hr) or the combination of PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr). 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 two weeks of the experiments, the PHY and SCO concentrations were based on the estimated weight of the animals one 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 two days before starting the training in the shuttlebox. In order 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 8 animals each were formed that showed no significant differences in any of the behavioural 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 five and ten days later.

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 ten 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 sc 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 x LD_{50} dose of soman. The sc LD_{50} dose of soman in guinea pigs is 24.5 µg/kg as determined before (Gordon and Leadbeater, 1977). All animals received atropine therapy (17.4 mg/kg im) 1 minute after soman. The symptomatology was closely observed during the first three hours by investigators unaware of the treatment and the lethality was determined at 24 and 48 hours.

TABLE 1

Test protocol of the 5 different treatment groups.

Treatment	Tests	n
subchronic PHY (0.025 mg/kg/hr)+ SCO (0.018 mg/kg/hr)	EEG, VER, shuttlebox, blood AChE (t=day 13) electrophysiology startle, blood AChE (t=day 7), receptor binding efficacy against soman, blood AChE (t=day 10) SCO plasma concentration	8 5 8 8 24
subchronic PHY (0.025 mg/kg/hr)	EEG, VER, shuttlebox, startle, receptor binding and blood AChE (t=day10) efficacy against soman, blood AChE (t=day 10)	8 5
subchronic SCO (0.018 mg/kg/hr)	receptor binding	8
acute PHY (0.4 mg/kg) + SCO (0.1 mg/kg)	efficacy against soman, blood AChE (t=day 10) SCO plasma concentration	8 8
subchronic vehicle (control)	EEG, VER, Shuttlebox, blood AChE (t=day 10) electrophysiology startle, blood AChE (t=day 7), receptor binding	8 4 8

Shuttlebox performance

An automated two-way shuttlebox, consisting of two equal compartments of 23x23x23 cm with rounded corners, connected by a photo-cell-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 ref. Philippens *et al.*, 1992a). 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 digitised 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 immobilised in a vertically mounted PVC tube (as for the startle response). Fast Fourier transformation (FFT), to obtain power spectra, was performed on line from 5 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 (50.000x), 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 (Hong and Chang, 1991) was added to the bath medium for at least one hour. After washout of μ-conotoxine recordings were started. Endplate 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 non-linear summation, assuming a reversal potential of -5 mV (McLachlan and Martin, 1981), 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 homogenised (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. Non-specific 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 fibre 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, Kd, Ki 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 (Johnson and Russell, 1975). 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 (Cintron and Chen, 1987). Guinea pig brain membranes were prepared as described earlier (Van Helden *et al.*, 1994). 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 mini-pump containing PHY (0.025 mg/kg/hr)+ SCO (0.018 mg/kg/hr). Plasma concentrations of SCO were also determined after single sc injection 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 s.c. injection with 0.1 mg/kg SCO, was 43 ± 9 nM (n=8).

Effects on behavioural and neurophysiological parameters

The measurements of possible behavioural and neurophysiological side effects were started two 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/hr) alone or PHY in combination with SCO (0.018 mg/kg/hr) 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 8 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 post-hoc analysis). An ANOVA analysis also showed a significant effect on the Kd ($F(3,32)=3.44$; $p=0.028$). A Newman-Keuls post-hoc analysis showed that the Kd in the PHY+SCO treated group was significantly lower than the Kd's in the PHY- and SCO-treated groups. However, no significant difference with respect to the control group was found.

TABLE 2

Muscarinic receptor binding after a 14 day treatment with PHY, SCO or PHY+SCO. The Bmax is given as a percentage of control value; the Kd is given in pM.

	Muscarinic Receptor binding		
Treatment	N	Bmax	Kd
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+

**significantly different from control value (Newman Keuls)*
+significantly different from PHY- and SCO treatment

Efficacy against a 3 x LD_{50} dose of soman

a) *Pretreatment with acute PHY+SCO (vehicle in Alzet® pumps) against 3xLD_{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 intoxication. Only one animal suffered from convulsions (lasting about 2 min) and dyspnoea. None of the animals died within one week after intoxication, 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.

b) Pretreatment with subchronic PHY+SCO against 3xLD$_{50}$ 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 38h, resp., 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).

c) *Pretreatment with subchronic PHY against 3xLD$_{50}$ 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 5 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 2 animals only showed a slight ataxia for a period of 11-17 minutes, starting 3 and 9 minutes after intoxication, resp. All animals were largely recovered about 30 min after intoxication and completely free of apparent symptoms 2 hours 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 as 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.

TABLE 3

% survival at 24h and 48h after a 3xLD$_{50}$ 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 3xLD$_{50}$ 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%

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 behavioural 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.

In an earlier study we already showed (Philippens *et al.*, 1996) that an acute dose of PHY, leading to similar levels of AChE inhibition as found in the present study, causes unacceptable behavioural 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 (Philippens *et al.*, 1996). 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 (Costa *et al.*, 1982; Wolthuis *et al.*, 1995). Indeed, in our electrophysiological experiments on diaphragms obtained from subchronically treated animals with PHY+SCO, a clear decrease of the quantal content was found which is in accordance with earlier findings regarding tolerance to the effects of DFP (Melchers and Van der Laaken, 1990), paraoxon (Thomsen and Wilson, 1988), and also to the carbamate neostigmine (Tiedt *et al.*, 1978). 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, e.g. the startle response, that were interpreted in an earlier study (Philippens *et al.*, 1996, Philippens *et al.*,1997) 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 (Lim *et al.*, 1987). The increased number of muscarinic binding sites after SCO treatment appears to be in agreement with the findings of Baskin *et al.* (1994). After (sub)chronic treatment with AChE-inhibitors, usually a down-regulation of muscarinic receptors is found (Costa *et al.*, 1982; Van Dongen and Wolthuis, 1989; Gazit *et al.*, 1981). However, Bhat *et al.* (1990) 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 (Albuquerque *et al.*, 1988). Furthermore, PHY has also a decreasing effect on the ACh-release that was found in the neuromuscular junction (Provan and Miyamoto, 1991).

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 (Lim *et al.*, 1988b; Harris *et al.*, 1989). Besides, PHY pretreatment is more effective than a pretreatment with PYR (Table 3). In 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 (Inns and Leadbeater, 1983).
In most of our experiments, PHY was combined with SCO. This was based on the results of Leadbeater (1985) as well as on our own earlier study showing that SCO could counteract at least some of the behavioural and neurophysiological side effects of PHY when given as one acute dose.
The dose of SCO we used (18 μg/kg/hr) led to a plasma levels of 45 nM, which is much higher than those found by Wetherell (1994), 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 non-linear kinetics of SCO. SCO is mainly excreted through metabolism into inactive metabolites (Ali-Melkkila *et al.*, 1993). Saturation of hepatic elimination may cause an extraproportional increase of plasma SCO levels. Indeed Wetherell (1994) 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 x 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 behavioural performance and the neurophysiological activity were not affected compared to the acute pretreatment of PHY (Philippens *et al.*,1996). 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 man, it is imperative that they are substantiated by similar findings in other animal species closer related to man. In this respect, the marmoset monkey seems to be the best alternative (Baker *et al.*, 1984; Ridley *et al.*, 1984; Wolthuis *et al.*, 1995).

Acknowledgements
The authors would like to thank J.J. Zijlstra, A.L. van der Laaken and B. Groen for their excellent technical assistance.

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Subchronic physostigmine pretreatment in marmosets: absence of side effects and effective against soman poisoning with negligible post intoxication incapacitation

Ingrid H.C.H.M. Philippens[1], Raymond A.P. Vanwersch[1], Bas Groen[1], Berend Olivier[2], Piet L.B. Bruijnzeel[1], Bert P.C. Melchers[1]

[1]*Dept. of Pharmacology, TNO Prins Maurits Lab (TNO-PML), Rijswijk ZH, The Netherlands.*

[2]*Dept. of Psychopharmacology, Rudolf Magnus Institute for Neurosciences, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands.*

Subchronic pretreatment with physostigmine (PHY) (0.0125 mg/kg/hr), leading to an acetylcholinesterase inhibition of 30%, caused no side effects when applied to marmoset monkeys. This was evident on behavioural parameters and on EEG and cortical Visual Evoked Response. Furthermore, this treatment regimen, followed by atropine as post intoxication therapy, protected the marmosets against lethality after a 2x LD_{50} dose of soman and resulted in negligible post intoxication incapacitation. These findings suggest that a sign free pretreatment with subchronic PHY should be able to protect man sufficiently against severe soman intoxication.

Toxicological Sciences. Submitted

Introduction

Nerve agents of the organophosphorus type inhibit irreversibly the enzyme acetylcholinesterase (AChE). This inhibition leads to accumulation of acetylcholine (ACh) resulting in central and peripheral cholinergic effects (Taylor, 1996). Protection against organophosphate (OP) intoxication may be achieved by an effective treatment regime. Pretreatment with a carbamate, that protects the AChE from attack by the OP compound, followed by a therapy with an anticholinergic drug seems to be most effective against the OP soman (1,2,2-trimethylpropyl methylphosphonofluoridate) in different animal species (Berry and Davies, 1970; Dirnhuber *et al.*, 1979; Gordon *et al.*, 1978; Inns and Leadbeater, 1983; Heyl *et al.*, 1980). The current concept of treatment upon acute OP intoxication is to apply several auto-injectors containing an oxime, atropine and diazepam. The current concept of pretreatment is the oral administration of pyridostigmine (PYR). A major drawback of the quaternary carbamate PYR is that it is unable to penetrate the central nervous system (CNS) to any significant extent and, therefore, cannot protect the brain against the intoxicating effects of OPs. Based on the lack of penetration of PYR in the brain experimental animals take many hours to recover (Inns and Leadbeater, 1983). Therefore, it is essential that a combination of pretreatment and treatment should prevent, or at least markedly reduce, nerve agent-induced decrements in human performance. For this reason physostigmine (PHY) has been proposed as an alternative for PYR (Leadbeater *et al.*, 1985). This compound possesses a tertiary nitrogen atom and does penetrate the brain and has been shown to be effective against OP intoxication. A significant protection against lethality after sarin or soman-intoxication was reported (Leadbeater *et al.*, 1985). However, a pretreatment not only must be effective against lethality and post-intoxication incapacitation, but also be devoid of side effects, especially when given for a long period of time. Since PHY has a short plasma half-life and a narrow therapeutic index (Somani and Khalique, 1986), a bolus injection of PHY is not a very realistic pretreatment procedure. A more chronic application should be considered and evaluated. In a previous study the presence of side effects after subchronically administered PHY were determined in guinea pigs (Philippens, *et al.*, 1998): subchronic application by osmotic mini-pumps of PHY caused no behavioural or neurophysiological side effects and was effective against a 3x LD_{50} dose of soman. In order to provide a firmer basis for this pretreatment in man, a similar type of study as in the guinea pig was undertaken in the marmoset, a non-human primate. Behavioural and neurophysiological test methods were used to determine the side effects of PHY and post-intoxication incapacitating effects after soman poisoning with 2x LD_{50}.

Methods

Animals

Adult Marmoset monkeys (Callithrix jacchus) of both sexes bred and raised at the Biomedical Primate Research Centre ((BPRC), Rijswijk, The Netherlands) were used. The animals were housed separately in cages (61 x 61 x 41 cm) in a room kept at 23-25°C and at a relative humidity >60%. In this room a 12 hour day and night cycle was maintained. Daily they were fed with rice, peanuts, fruit, boiled egg, baby crackets, sunflower seeds, honey, broad bean, Karvan cevitam^{R} and pellet chow after training or testing. Water was available ad libitum. The here described experiments received prior approval by an independent ethical committee.

Drug solutions and implantation of osmotic mini-pumps

Physostigmine (eserine) was obtained from Sigma (St.Louis, U.S.A.); Atropine Sulphate was obtained from ACF (Amsterdam, The Netherlands); Soman (O-pinacolyl methylphosphonofluoridate) was synthesised at the Prins Maurits Laboratory TNO (Dr H.P.Benschop). Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.5 µl/hr (Model 2002, Alza Corp., Palo Alto, CA, USA) were used to deliver PHY dissolved in a vehicle. The osmotic mini-pump was implanted subcutaneously on the back between the shoulders of the animal under ketamine anaesthesia (20 mg im/animal). The wounds were sutured with silk. The dose of PHY used was 0.0125 mg/kg/hr. In a pilot study this dose offered a therapeutically relevant AChE inhibition. The PHY concentration was based on the body weight of the animals before implantation. The vehicle consisted of 20% propylene glycol, 10% ethanol and 70% water (1 part glacial acetic acid in 2000 parts distilled water).

Study design

The here-described study was performed in two different treatment groups of animals. One group received subchronic pretreatment with PHY (0.0125 mg/kg/hr; for 12 days) before soman (2x LD_{50}) intoxication (n=6: monkeys CJ, GW, DP, GT, GU and GV). The other group served as a control group, not receiving PHY pretreatment (n=3: monkeys FM, DJ and FH). Both groups received atropine sulphate (5 mg/kg; im) therapy (Hamilton and Lundy, 1989; Van Helden *et al.*, 1992) one minute after soman intoxication. The LD_{50} dose of soman (applied subcutaneously) used was 9 µg/kg (Dirnhuber *et al.*, 1979). The appearance of side effects after PHY pretreatment and the protection of this pretreatment against lethality and post-intoxication incapacitation after soman poisoning were tested.

Training of the animals in the hand-eye co-ordination task started 5 weeks before the start of this study. Electrodes for the measurement of EEG and Visual Evoked Response (VER) were fitted four days before implantation of the Alzet® mini-pumps. In order to obtain control values all the parameters (AChE activity, body weight and temperature, hand-eye co-ordination, bungalow performance, startle reflex, EEG and VER) were registered / determined before implantation of the Alzet® minipumps. The hand-eye co-ordination was only performed in five monkeys (monkey GW did not reach the critical performance level during training). The neurophysiological tests (EEG and VER) were only performed on 4 monkeys (CJ and GW were excluded, since there were problems with their electrodes).

The day Alzet® mini-pumps were implanted was called day 0. Three days after implantation behavioural and neurophysiological tests were started: these were repeated at days six and ten. Body weight and temperature were measured and blood samples were collected at days 3, 5, 7, 10 and 12. At day 12 the animals were intoxicated with 2 x LD_{50} soman (sc) 30 minutes after removal of the pumps. Twenty minutes after removal of the Alzet® mini-pump a blood sample was drawn for the determination of blood AChE. One minute after soman injection all the animals received an im injection with atropine sulphate. After soman intoxication the animals were observed for clinical signs like attention, salivation, tremors, and convulsions. One hour after soman poisoning the body temperature was registered and a blood sample was taken to measure the AChE inhibition. After the intoxication symptoms had disappeared the same behavioural and neurophysiological tests were carried out. After the first two monkeys

were intoxicated with soman it appeared that they were in such a good condition that we decided to perform the behavioural and neurophysiological tests already one hour after intoxication in the remaining four animals. The tests were repeated at days 13, 17, and 19. In the control animals the clinical symptoms of intoxication progressively became worse till the animals deceased. These animals could not be tested in any of the tests.

Behavioural tests in marmosets

a) *Hand-eye co-ordination test*

The hand-eye co-ordination was performed with an automated robot-guided apparatus according to Wolthuis *et al.* (1995) using positive reinforcement as a motivating stimulus (small pieces of marshmallow). A robot is situated behind a test panel provided with two windows (8 cm wide and 5 cm high). These windows can open and close through a pneumatically driven and vertically sliding door. For the hand-eye co-ordination task only the left window was used. In front of the test panel the test cage (32.5 x 24 x 24 cm) in which the marmoset is placed is situated. The side of the test cage directly in front of the panel consists of horizontal stainless steel rods, spaced far enough apart to allow the animal to reach its arm at full length through the window. The robot held an 8.5 cm long suction tube. For each trial the robot turns to a plateau containing the rewards, sucks one reward onto the tip of the tube and then moves it into the starting position behind the test panel. The presence of the reward at the tube is checked by a pressure detector that also registers the time needed for removal during the trial. A photocell-monitored trough on the inner side of the test-panel registers the rewards that are not properly retrieved by the animal through the window into the test cage. Infrared detectors within the windows allow the registration of successful attempts of the animal to grasp a reward. With this system three types of trials are performed: one using a non-moving reward in the middle of the window, one using a slow horizontally moving reward (0.04 m/s) and one using a fast horizontally moving (0.08 m/s) reward from the left to the right side of the window. The animal is allowed one minute to grasp the non-moving reward. Each type of trial is performed 14 times in one session. At the beginning of each trial a sound signal is presented, intended to alert the animal. Immediately thereafter the window opens. At that point of time the suction tube is in the ready position in the non-moving trials and starts in the moving trials to move to the other position. A "hit" is registered when the animal successfully retrieves the reward from the suction tube. The number of attempts and failures are also registered. The percentage of correct hits is used as criterion to judge the performance of the animal.

b) *Bungalow test*

The bungalow test is an automated test system that allows the registration of the activity/exploration of the marmoset. This equipment consists of four equal compartments of 23x23x23 cm that are all connected with each other by 6 photo-cell-guarded PVC-tubes. The animals can freely move and change from one compartment to the other during a 20 minute session (for details; see Wolthuis *et al.*, 1994). The motor activity is expressed as the number of compartment changes in this time period.

c) *Auditory startle response*

In this test the stretching movement of the legs is used to reflect the reaction of the animal on a startle signal (Davis, 1992). For this test the animals are exposed to 20 auditory startle pulses (120 dB, pink noise, 20 ms) while standing on a platform in a PVC-tube (diameter 17.5 cm, length 26 cm). The startle response of 200 ms duration is measured by a transducer connected with the platform, registering the force exerted by the animal upon presentation of the stimulus. The AD converter of an IBM-compatible PC digitised the responses. The amplitude and latency of the startle response are registered and used to measure the motor force of the startle reflex.

Neurophysiological measurements

Under ketamine anaesthesia a silver electrode is placed, by using a stereotact, into a small hole in the skull above the visual area (3 mm lateral to the sutura sagitalis and 5 mm caudal from intra-aural), leaving the dura mater intact. A reference electrode is placed over the sinus. Both electrodes are connected with a plug and fixed on the skull with dental cement. During the test a transmitter is connected to the plug for telemetric registration of the EEG and VER. All EEG signals were amplified (50.000 x), filtered (between 0-30 Hz for EEG and 0-500 Hz for VER) and fed into the AD converter of an IBM-compatible PC; sampling frequency was 50 Hz for EEG and 1 kHz for VER.

a) *EEG registration*

Fast Fourier transformation (FFT), to obtain power spectra, is performed from 5 randomly chosen EEG epochs of 12 s out of a total recording time of 3 min. The obtained power spectra are subdivided into 8 frequency classes (0.8-2, 2-3.5, 3.5-5.5, 5.5-7.5, 7.5-10, 10-12.5, 12.5-18, 18-25 Hz). The total power (V^2) of the different frequency classes are used for the evaluation of the brain activity.

b) *VER registration*

For registration of the VER the animals receive 30 light stimuli with a time interval of 2 sec $\pm$ 20%. Following the stimuli EEGs are registered for 250 ms and the responses averaged. For evaluation of effects the latencies and amplitudes of the positive (P1, P2, P3) and negative (N1, N2, N3) peaks were measured and compared with the baseline values.

Determination of AChE-activity

The sole of the foot of the marmoset was punctured using an Autoclix lancet (Boehringer, Mannheim, Germany). Blood samples (5 µl) were taken and immediately mixed with 50 µl 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 (Jonsons and Russell, 1975). 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.

Statistics

A repeated analysis of variance (ANOVA) was used to assess statistical significance in the side effects results, a two way ANOVA followed by a Newman-Keuls post-hoc test in the

behavioural incapacitation results and a paired T-test in the neurophysiological test systems. For the symptomatology after soman intoxication a Fisher exact probability test was used. In all tests p values < 0.05 were considered significant.

Results

In this study three aspects of subchronic PHY pretreatment were studied: a) the side effects during subchronic administration, b) the protection against lethality induced by 2x LD_{50} soman, and c) the protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman.

The side effects during subchronic administration of PHY

Body weight and temperature after PHY

Body weight was not affected by the subchronic administration of PHY. During the pretreatment period of subchronic PHY a small not significant decrease was observed in the rectal temperature from 39.3 to 38.7°C. These rectal temperature changes are within the normal daytime range for such monkeys that varies between 38.5°C and 40.0°C.

Blood AChE inhibition after PHY

The mean blood AChE-inhibition of the subchronic PHY-treated animals (n=6), measured as a percentage of their control value before osmotic pump implantation, at days 3, 5, 7, 10, and 12 after osmotic pump implantation were: 35.4 ± 5.3 %, 36.4 ± 4.5 %, 26.8 ± 4.1 %, 20.9 ± 4.8 %, and 30.7 ± 7.5 % respectively.

Side effects on behavioural parameters

In Fig. 1 the side effects of ten days subchronic PHY administration on the behavioural parameters are shown. In the hand-eye co-ordination performance a small, but not significant, decrease was found in the total number of "hits" during the pretreatment period (a repeated ANOVA analysis showed P=0.13). This decrease was mainly caused by monkeys DP and CJ. DP had a high control performance of 40 hits in 42 trials. After pump implantation she scored 31, 29 and 18 hits respectively.

The exploration/activity of the animals, measured by the number of compartment changes in the bungalow test, was also not affected by subchronic PHY administration (p=0.98). This was mainly due to the variation of exploration between the animals: some animals showed a decrease and some an increase in their activity.

In the startle reflex test neither the amplitude of the response (Fig. 1) or the latencies were affected by subchronic PHY administration compared with the control values (at all test points a repeated ANOVA analysis showed P=0.87 for all the parameters).

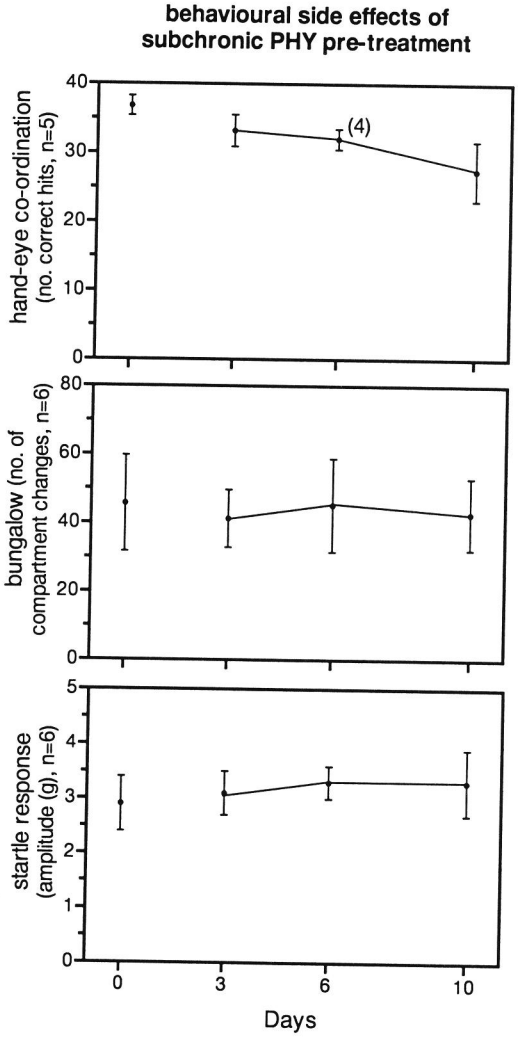

FIG 1: Behavioural side effects before (control value: day 0) and during PHY pretreatment (0.0125 mg/kg/hr) at 3, 6, and 10 days after osmotic mini-pump insertion.
A: Number of correct hits in the hand-eye co-ordination task (mean ±SEM, n=5).
B: Number of compartment changes in the bungalow task (mean ±SEM, n=6).
C: Amplitudes of the startle response (mean ±SEM, n=6).

Side effects on neurophysiological parameters

The total band power (V^2) in the different frequency classes of the subchronic PHY treated animals showed no difference compared with their control value (not shown). The VER consists of three positive and three negative peaks. The amplitude and the latency of each peak were measured. The data were averaged and compared with the control value (Fig.2). The latencies were not affected in the animals receiving PHY compared with their control value (at all registration points a paired T-test analysis showed $P>0.05$ for all the different

parameters in the EEG and the latencies in the VER). Only the amplitude of peak P1 at day 3 after osmotic pump implantation significantly decreased (baseline value: 108.3 ± 38.2 μV, day 3: -45.3 ± 40.7 μV (p=0.03), day 6: 27.8 ± 63.7 μV, day 10: 25.0 ± 88.6 μV).

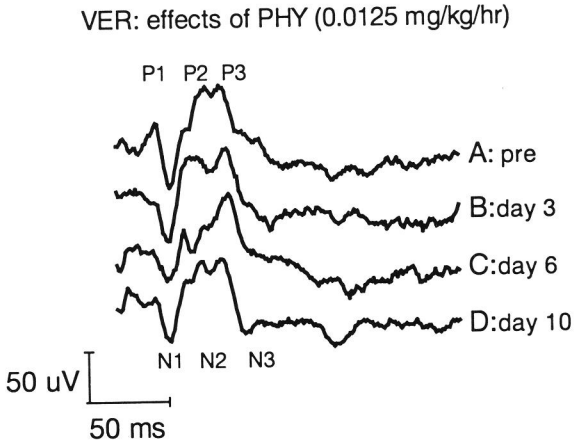

FIG. 2: The averaged VERs: negative peaks are plotted downwards, positive peaks upwards. Control value: A= before osmotic mini-pump insertion (n=4). During subchronic PHY pretreatment (0.0125 mg/kg/hr): B= 3, C= 6, and D= 10 days after osmotic mini-pump insertion (n=4)

The protection against lethality induced by 2x LD_{50} soman

Body weight and temperature after PHY

After osmotic pump removal and one hour after soman intoxication and atropine therapy (day 12) the rectal temperature did not further decrease (38.6°C ± 0.21; n=6) whereas in an animal that only received atropine therapy after soman intoxication it did (32.6°C; n=1). The body weight was not affected by soman intoxication measured 24 hours after the intoxication.

Blood AChE inhibition after PHY

The mean blood AChE-inhibition of the PHY-treated animals, measured at day 12 after osmotic pump removal and just before soman intoxication, was 28.3 ± 3.1 % (n=6), and one hour after soman intoxication amounted 90.5 ± 1.8 % (n=6) of the control value. This in contrast with the unpretreated control animals were the AChE level was inhibited down to 97.9 ± 2.1 % (n=3). Shortly thereafter these animals deceased.

Protective efficacy of PHY pretreatment against a 2x LD_{50} dose of soman

All animals pretreated with subchronic PHY and treated with atropine against 2x LD_{50} soman (n=6) survived the intoxication, whereas all the animals only treated with atropine died after intoxication. The monkeys (FM, DJ and FH) deceased at 2, 1.5 and 1 hours after soman intoxication. The difference in survival was significant (Fisher exact probability test, $p<0.05$, two-tailed).

The protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman

Post-intoxication incapacitation symptoms after 2x LD_{50} soman

The animals pretreated with subchronic PHY and treated with atropine against 2x LD_{50} soman (n=6) appeared to be in a good condition. They only showed mild tremors and some ataxia, lasting from 10 min after intoxication till about 50 min after intoxication. During this time the animals stayed alert and very active. Monkey GU (male) even showed sexual activity towards GV (female) that started 45 minutes after soman poisoning. This interest was mutual. All animals largely recovered about 1 hour after intoxication and were judged to be able to perform behavioural tests. Two hours after soman they were completely free of apparent symptoms and started eating.

The animals of the control group (n=3), only treated with atropine, were in a much worse condition compared to those pretreated with subchronic PHY. All animals showed severe tremors and convulsions lasting for periods of 10 to about 20 min., followed by a period of muscle fasciculations. The first symptoms started within 10 minutes after intoxication. During the convulsive activity the animals started to suffer from dyspnoea that lasted until they died. Shortly after the convulsions all the animals became comatose. The clinical symptoms of the animals in this group were significantly worse compared with those pretreated with subchronic PHY (Fisher exact probability test, $p<0.05$, two-tailed).

Post-intoxication incapacitation effects on behavioural measurements

The post-intoxication incapacitation effects on the behavioural parameters after soman intoxication are showed in Fig. 3. One and a half hours after soman intoxication the animals performed different in the hand-eye co-ordination task. Two monkeys, both male, showed a very good performance (GT: 31 and GU: 38. Their control values were 36 and 38 respectively) In contrast the female monkeys performed very poorly in this task (DP: 13 and GV: 0). In the test cage of DP three marshmallow rewards she obviously had spit out were found after the test. It could be that the motivation was decreased by queasiness, but after the tests all the animals started to eat their normal food. One day later monkey GV was recovered completely in this task (performance score of 40) whereas monkey DP was still performing poor (score of 7). The other animals maintained performing well. Five days after soman intoxication (day 17) monkey DP was back to a normal performance (score of 37).

One hour after soman the animals were tested in the bungalow test where no effect on the activity was observed; the mean number of compartment changes was found to be 48.8 ± 13.3 (n=4). Twenty four hours after soman (day 13) the mean number of compartment changes was not significantly decreased compared with one hour after soman to 35 ± 3.7 (n=4; GT, GU, GV, DP) and also not when compared with the baseline value (an ANOVA analysis showed $P>0.05$).

In the startle reflex test a, not significant, increase of the startle response amplitude was observed one-hour after soman intoxication. Twenty-four hours later (day 13) this tendency had disappeared (2.5 ± 0.3; n=6).

Post-intoxication incapacitation effects on neurophysiological measurements

Two hours after soman intoxication the EEG and VER were registered. The total power of the different frequency classes from the EEG is summarised in Table 1. In all the animals a shift

of the power towards the higher frequencies was noticed after soman intoxication. This remained present during the study and was only found significant in the first frequency band (0.8-2.0 Hz) at twenty-four hours (day 13) and one week (day 19) after soman intoxication.
The amplitude and the latency of each peak were measured, averaged and compared with the control values. For all test points no effect was found on the latency of the VER peaks after 2x LD_{50} soman intoxication in PHY pretreated animals compared with their control (at all registration points $P>0.05$). The amplitudes of the VER peaks were also not affected: only the amplitude of peak P3 at twenty four hours after soman intoxication was significantly decreased compared with the control value ($p=0.04$) (Table 2). Thereafter this effect disappeared.

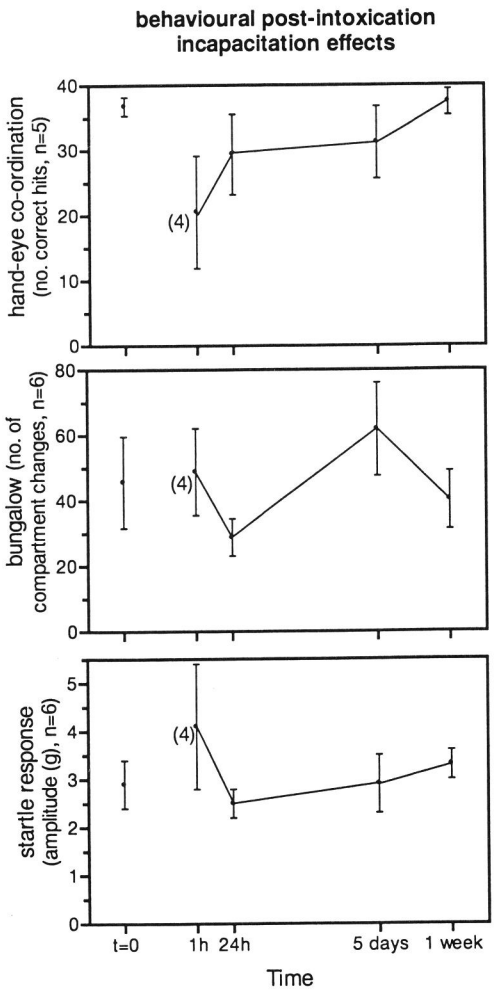

FIG. 3: Behavioural post-intoxication incapacitating effects before PHY pretreatment (control value: day 0) and after 2x LD_{50} soman intoxication (followed by atropine 5 mg/kg im) at 1-1.5 hours and 1, 5, and 7 days after intoxication. At 1-1.5 hours after intoxication the behavioural tests were performed with n=4 animals.
A: Number of correct hits in the hand-eye co-ordination task (mean $\pm$SEM, n=5). B: Number of compartment changes in the bungalow task (mean $\pm$SEM, n=6). C: Amplitudes of the startle response (mean $\pm$SEM, n=6).

TABLE 1

Effects on EEG expressed as the mean (± SEM) (n=4) band power (V^2) of the different frequency classes measured before the start of the study (day 0) and after 2x LD_{50} soman intoxication followed by a therapy of atropine in PHY pretreated animals.

Test	power (V^2 *(±SEM)*) of the different frequency classes (Hz)							
Day	0.8-2	2-3.5	3.5-5.5	5.5-7.5	7.5-10	10-12.5	12.5-18	18-25
0	307.7	242.8	225.2	195.2	197.0	158.7	247.5	242.0
	(54.0)	*(58.0)*	*(52.7)*	*(71.8)*	*(88.8)*	*(40.0)*	*(88.2)*	*(75.7)*
12	159.7	202.5	159.7	110.8	121.5	152.2	361.8	298.0
	(35.8)	*(66.3)*	*(24.4)*	*(17.5)*	*(16.2)*	*(17.8)*	*(38.3)*	*(19.4)*
13	*143.3	130.8	124.7	101.3	104.5	178.8	354.8	370.2
	(32.3)	*(15.5)*	*(11.2)*	*(17.3)*	*(19.1)*	*(22.9)*	*(16.2)*	*(29.8)*
17	155.0	147.5	165.7	107.0	124.3	182.3	380.0	337.7
	(51.7)	*(21.7)*	*(23.2)*	*(19.1)*	*(15.6)*	*(25.8)*	*(12.7)*	*(42.7)*
19	*98.2	123.3	124.0	93.8	127.8	138.8	399.8	351.3
	(16.3)	*(23.1)*	*(18.5)*	*(11.4)*	*(25.4)*	*(21.1)*	*(0.3)*	*(34.3)*

Note. At day 12 the EEG was registered 2 hours after soman intoxication
*Significantly different from control value ($p<0.05$).

TABLE 2

Mean (±SEM) amplitudes (n=4) of the positive (P1, P2, P3) and negative (N1, N2, N3) peaks of the VER measured before the start of the study (day 0) and after 2xLD_{50} soman intoxication followed by a therapy of atropine in PHY pretreated animals (days 12, 13, 17, 19)

	Amplitude (mV) (±SEM) of the VER peaks											
Day	P1		N1		P2		N2		P3		N3	
0	108	(38)	-413	(58)	380	(22)	241	(85)	415	(66)	-169	(120)
12	20	(113)	-405	(96)	344	(169)	97	(170)	358	(144)	-248	(114)
13	63	(45)	-442	(130)	245	(110)	69	(150)	*100	(97)	-427	(75)
17	173	(113)	-415	(135)	288	(130)	101	(84)	238	(136)	-296	(71)
19	108	(119)	-387	(174)	407	(230)	151	(125)	262	(125)	-213	(55)

Note. At day 12 the VER was registered 2 hours after soman intoxication
*Significantly different from control value ($p<0.05$).

Discussion

In this study the side effects, the protective efficacy against soman (2x LD_{50}) induced lethality and the post soman intoxication incapacitation after subchronic pretreatment with physostigmine (PHY) were investigated in marmosets.
After a subchronic pretreatment with PHY (0.0125 mg/kg/hr) for 12 days at a therapeutic dose, resulting in a blood AChE inhibition of 30%, no behavioural or neurophysiological side effects could be observed in the tests performed. In an earlier study in guinea pigs, following almost the same protocol as in the present study, we already demonstrated (Philippens *et al.*, 1998) that subchronic PHY caused no behavioural and neurophysiological side effects. In contrast, in a previous investigation it was shown that a single dose of PHY (0.6 mg/kg), leading to a comparable AChE inhibition, caused unacceptable side effects in this animal species (Philippens *et al.*, 1996). This was also confirmed in the marmoset (Wolthuis *et al.*, 1995). A single dose of PHY 0.02 mg/kg (im) that caused an inhibition of the blood AChE activity >20% resulted in a significantly high performance decrement in the hand-eye co-ordination test. Obviously, the plasma concentration of the PHY after a bolus injection will be higher when compared with a subchronic infusion and may be responsible for the observed side effects. Another explanation for the lack of side effects after subchronic PHY administration could be the well-known phenomenon of development of tolerance for AChE-inhibitors (Costa *et al.*, 1982; Wolthuis *et al.*, 1990; Lim *et al.*, 1987; Van Dongen and Wolthuis, 1989). Taken together, this study and a previous one in guinea pigs indicate that subchronic administration of PHY is the route of choice to prevent the development of unwanted side effects.

The here presented results also demonstrate that a subchronic pretreatment with PHY efficiently protects marmoset monkeys from soman (2x LD_{50}) induced lethality without causing severe post-intoxication incapacitation symptoms. The efficacy against soman induced lethality in this study is similar to that found in a previous study in guinea pigs (Philippens *et al.*, 1998). This finding is in accordance with other studies: guinea pigs continuously treated with PHY via osmotic mini-pumps were protected against soman-induced toxicity (Lim *et al.*, 1988b). In contrast, these investigators did not observe this protection after a single dose PHY pretreatment. The lack of convulsions and the relative good clinical condition of the marmosets after soman intoxication in this study, suggest that the protection after subchronic PHY pretreatment in the marmoset is much more effective than in the guinea pig. This discrepancy can be attributed to subtle species differences in the AChE-molecule. This may result in different reactivation kinetics of AChE after reversible binding of PHY to AChE. The fact that pretreatment with a carbamate, combined with post-intoxication atropine therapy, protects efficiently against soman poisoning has already been reported for different animal species. However, there exists a marked species variation in the efficacy of this treatment regime (Berry and Davies, 1970; Gordon *et al.*, 1978). Obviously non-human primates are very well protected in this way. This could indicate that man would also benefit greatly from such a treatment regime. Based on the here presented results one is inclined to conclude that subchronic pretreatment with PHY for 12 days (together with post-intoxication atropine therapy) even offers a better protection against 2x LD_{50} soman intoxication than a therapy with an oxime (combined with atropine). This observation is based on previous findings: marmoset monkeys, intoxicated with 2x LD_{50} soman followed one

minute later by a therapy of HI-6 and atropine sulphate (0.5 mg/kg im) show much worse post-intoxication symptoms than reported here (Busker *et al.*, 1996). In that study, the animals remained irresponsive to their environment at least for one day and were not able to move, eat or drink by themselves for three to four days. In one animal it took even 18 days before she started to eat by herself. A possible explanation for the lower efficacy of oxime therapy against soman poisoning in marmosets could be the ageing phenomenon of the soman-AChE complex. Soman-inhibited AChE of marmosets and man "ages" with a $t_{1/2}$ of 1-1.5 min. (Talbot *et al.*, 1988). This means that only a small amount of soman-inhibited AChE can be reactivated after an acute intoxication since AChE is already "aged". Therefore, a therapy with an oxime may be less effective than a good pretreatment with PHY. Busker *et al.*(1996) also reported a difference in response between male and female marmoset monkeys. The females needed more time to recover from soman intoxication completely. In this study we also noticed a sex difference. After soman intoxication both female monkeys showed a very strong performance decrement in the hand-eye co-ordination task while the performance in the male monkeys was not affected. Therefore, it seems that females are more sensitive to OP intoxication than males (Sket, 1993). Thus, subchronic PHY pretreatment in combination with atropine therapy seems a very effective protection against 2x LD_{50} soman intoxication. Whether this is also valid for other OPs has to be further elucidated. However, based on literature data (Harris *et al.*, 1991) one should expect this to be the case.

Using the combination of subchronic PHY and atropine sulphate therapy not only survival improved also post-intoxication incapacitation was hardly present. One hour after soman intoxication the animals were already able to perform the behavioural tests. The male monkeys even showed a normal hand-eye co-ordination. Furthermore, all the monkeys were very alert and active what came also to expression in the bungalow task: a normal performance of motor activity was measured. There was even a tendency of an increase of the motor activity. This increase may be the result of stimulation of ACh receptors by increased amounts of ACh in the synaptic cleft. At that time the AChE inhibition amounted 90.5%. Also a not significant increase of the amplitude of the startle response was observed. This effect can not be due to AChE inhibition and ACh accumulation. It has been reported that most anti-ChE drugs failed to increase the startle reflex (Davis, 1982). Presumably direct effects on ACh receptors are involved in this effect (Philippens *et al.*, 1997). In the EEG power spectrum a shift from the lower towards the higher frequencies was found that also can be explained by the stimulation of ACh receptors. Delta activity may be associated with behavioural impairment (Vanderwolf, 1973). A decrease of this slow wave activity suggests an increase of the animal's activity. This is in accordance with our findings. In a previous study with rats an increase of the Delta activity was found one hour after 0.5x LD_{50} soman intoxication (Wolthuis *et al.*, 1991). Furthermore, a high degree of EEG synchronisation was observed around 8 Hz twenty-four hours after soman. At this frequency no effect was found in this study. This can be explained by the activity of the animals at that time. Frequencies around 8 Hz represents "walking" behaviour in rats. In the study of Wolthuis *et al.* (1991) the rats were forced to walk in a wheel while the EEG was recorded. In this study the animals were not forced to any active behaviour. The absence of the increase of the Delta activity suggests that PHY pretreatment counteract the effects of soman on the EEG. The increasing tendency of the high frequencies is hard to explain. One week after soman intoxication this

effect was still present. This suggests that a permanent change may have occurred in the brain. The impact of this change in EEG activity on long-term neurological effects needs further investigation. On the other hand, no effects of physiological importance on the VER were found. This finding is in accordance with other studies (DeBruyn *et al.*, 1991). They reported that PHY pretreatment combined with a treatment of mecamylamine (a nicotine receptor antagonist) and atropine reduced the effect of soman on the VER in the cat.

In conclusion, subchronic treatment with PHY seems to be a good alternative for the current PYR pretreatment, in particular since this pretreatment shows in the tests used no side effects, protects more efficiently against 2x LD_{50} soman intoxication and thereby leads to less post-intoxication incapacitation effects in marmoset monkeys. Although the here presented study strongly favours the use of subchronic PHY pretreatment, as an effective protection against soman intoxication, several points need consideration. When this pretreatment is going to be applied to man a continuous stable plasma level of PHY should be realised. For practical everyday use another route of administration than chronic infusion via osmotic mini-pumps should be developed. This can only be realised by a similar type of equipment as the osmotic pump. In this respect, transdermal application has to be considered. Irregularities in the plasma level may result in less effective protection. Furthermore, during the first stage of the subchronic PHY pretreatment when a certain plasma level has to be reached, protection may be less adequate. This can be reached by an initiation dose of PHY at the start of the pretreatment period. In that case the combination with an anti-cholinergic drug like scopolamine is recommended. This allows a quicker achievement of a sign-free protective plasma level of PHY. In our opinion one should aim to reach a steady-state plasma level within 48 hours. In that case one should further investigate how well one is protected during that first 48 hours of PHY application. This will be the purpose of future investigations.

7

Scopolamine augments the efficacy of Physostigmine against Soman poisoning in Guinea Pigs

Ingrid H.C.H.M. Philippens[1], Bert P.C. Melchers[1] ,
Berend Olivier[2], Piet L.B. Bruijnzeel[1]

[1]*TNO Prins Maurits Lab (TNO-PML), Research Group Pharmacology, Rijswijk ZH, The Netherlands.*

[2] *Dept. of Psychopharmacology, Rudolf Magnus Institute for Neurosciences, Faculty of Pharmacy, Utrecht University, Utrecht, The Netherlands.*

The efficacy of the subchronically administered cholinesterase-inhibitor physostigmine (PHY) (0.025 mg/kg/hr) either with or without the muscarinergic receptor antagonist scopolamine (SCO) (0.018 mg/kg/hr) in counteracting soman-induced lethality and incapacitation were determined in guinea pigs. This was tested in animals that either received atropine sulphate (AS, 17.4 mg/kg im) or no post intoxication therapy. Behavioural and neurophysiological readout systems were used to measure post-intoxication incapacitation. Only the pretreatment with PHY alone did not offer any protection against 2x LD_{50} soman intoxication. Animals that received the complete treatment (PHY+SCO+AS) did not show any abberations in the performance of learned behaviour. The use of AS after soman intoxication resulted in an increase of the startle response, whereas the addition of SCO to the pretreatment led to a more persistent duration of the effect in time. In case one has to rely completely on the pretreatment the addition of SCO to PHY is life-saving. However, some post-intoxication incapacitation is still present. Therefore, the pretreatment regime may perhaps further be improved by the addition of a nicotinic antagonist.

Pharmacol. Biochem. Behav. Submitted

Introduction

The toxicity of organophosphorus (OP) compounds is known to be due to their acetylcholinesterase (AChE) inhibition in the synaptic cleft (Taylor, 1996). Protection against this type of intoxication can be achieved by pretreatment with a carbamate that inhibits the enzyme AChE in a reversible manner (Lennox *et al.*, 1985). Their prophylactic activity in reducing the toxic effects of the OP soman has already been shown (Berry and Davies, 1970; Lennox *et al.*, 1985). Currently, the carbamate pyridostigmine (PYR) containing a quaternary nitrogen is used as a pretreatment against OP intoxication. However, due to the quaternary nitrogen PYR can hardly pass the blood-brain barrier. Therefore, PYR only protects peripheral AChE. On the other hand, OPs easily penetrate into the brain because of their highly lipophilic nature, thereby causing both peripheral and central toxicity. For this reason a pretreatment against OP intoxication should not only protect the peripheral compartment but also the brain. Therefore physostigmine (PHY) has been proposed as an alternative for PYR (Leadbeater *et al.*, 1985). Leadbeater *et al.* reported PHY to be very effective against sarin or soman-intoxication. The centrally active carbamate PHY (containing a tertiary nitrogen) protects more effectively against soman intoxication than PYR (Harris *et al.*, 1984; Solona *et al.*, 1990). Rats pretreated with PHY recovered completely from soman intoxication within hours, whereas in rats pretreated with PYR symptoms lasted for one week (Harris *et al.*, 1984). Recently we have been able to confirm these data in guinea pigs (Philippens *et al.*, 1998). Guinea pigs pretreated with PHY appeared to be protected much better against soman induced lethality than those pre-treated with PYR. However, a pretreatment not only should be protective, it also should be devoid of side effects. Most of the side effects of the PHY pre-treatment are due to AChE inhibition in the central nerve system (CNS). These effects can be counteracted by the muscarinic receptor antagonist scopolamine (SCO) (Leadbeater *et al.*, 1985). Indeed, the addition of SCO to PHY antagonised the side effects in a memory task and in the EEG after single dose administration of PHY to guinea pigs (Philippens *et al.*, 1996). When PHY was applied subchronically no side effects were observed in both guinea pigs and marmoset monkeys (Philippens *et al.*, 1998; Philippens, Chapter 6). When PHY is applied subchronically (via an osmotic mini pump) SCO may not be necessary as a supplement to PHY therapy. However, the addition of an anti-muscarinic drug may improve the efficacy of PHY pretreatment (Harris *et al.*, 1980; Leadbeater *et al.*, 1985; Deshpanade *et al.*, 1986). The question addressed in this study therefore was: is additional therapy with SCO necessary to improve the protective efficacy of the pretreatment against intoxication with 2 x LD_{50} soman or does an adequate pretreatment regime offer sufficient protection by itself? Also we addressed the question whether the addition of SCO reduced the post intoxication incapacitation effects. These questions are relevant because in case of nerve gas exposure in a war situation soldiers have to inject themselves with a post intoxication therapy. One can imagine situations that soldiers are deprived of or are not capable of using this type of therapy. In that situation they have to rely completely on the pretreatment received. Behavioural and neurophysiological test methods were used to determine the post intoxication incapacitation effects of the various treatment regimes including or excluding SCO.

Method

Animals

Male Dunkin-Hartley albino guinea pigs CrL:(HA)BR (Charles River) with an initial body weight of 400-450 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. The here described experiments received prior approval by an independent ethical committee.

Drug solutions and implantation of osmotic mini-pumps

Physostigmine (eserine) and scopolamine bromide were obtained from Sigma (St.Louis, U.S.A.), atropine sulphate was obtained from ACF (Amsterdam, The Netherlands), soman (O-pinacolyl methylphosphonofluoridate) was synthesised at the Prins Maurits Laboratory TNO (Dr H.P.Benschop).

Alzet® Osmotic Mini-pumps with a constant delivery rate of 0.5 µl/hr (Model 2002, Alza Corp., Palo Alto, CA) were used to deliver either the vehicle, PHY (0.025 mg/kg/hr) or the combination of PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr). This dose of PHY offers the recommended blood-AChE inhibition of about 35 % (Philippens *et al.*, 1998). SCO (0.018 mg/kg/hr for a period of ten days) leads to a SCO plasma concentration of 45 nM (Philippens *et al.*, 1998). This was comparable with the level found after a single sc injection of 0.1 mg/kg SCO (43 nM). This SCO plasma concentration did not lead to side effects on shuttlebox performance and on the startle reflex, and antagonised some of the PHY induced side effects (Philippens *et al.*, 1992a, 1996). 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 dissolved in the vehicle. Because the animals gain weight during the two weeks of the pretreatment period, the PHY and SCO concentrations were based on the estimated weight of the animals one 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 anaesthesia. The wounds were sutured with woundclips.

Study design

The here-described study was performed in four different treatment groups of animals (n= 6 or 7 animals/group). Two groups were pretreated with PHY (0.025 mg/kg/hr) subchronically and two groups with the combination of PHY (0.025 mg/kg/hr) and SCO (0.018 mg/kg/hr). One group of each set of the different pretreatment groups received a post intoxication therapy with atropine (AS) (17.4 mg/kg im) one minute after a dose of 2x LD_{50} soman intoxication (day 11) (PHY/AS and PHY/SCO/AS groups). The remaining two groups did not receive any therapy after soman intoxication (PHY and PHY/SCO groups). The LD_{50} dose of soman (subcutaneously) used was 24.5 µg/kg (Gordon and Leadbeater, 1977). The protection of the different treatment regimes against lethality and post-intoxication incapacitation after soman poisoning was tested. Furthermore the pretreatment with PHY or PHY/SCO was checked for the occurrence of side effects on behaviour and neurophysiology (see below).

After the animals were trained in the shuttlebox, electrodes for the measurement of EEG and visual evoked response (VER) were fitted. In order to obtain control values, the body weight, blood-AChE activity, shuttlebox, startle response, EEG, and VER were registered /

determined before implantation of the Alzet® osmotic mini-pumps. Subsequently, based on the obtained results four matched subgroups of 6 animals each (n= 7 in the PHY/AS group) were formed that showed no significant differences in any of the behavioural tests. Thereafter, Alzet® osmotic pumps, containing either vehicle with PHY or a combination of PHY/SCO, were implanted. This was called day 0.

TABLE 1

Test protocol of all treatment groups.

Test days	Blood AChE activity	Body weight	Shuttlebox	Startle response	EEG and VER
3	X		X	X	
4		X			
5					X
7	X	X	X	X	
11	XXX	X	X	X	X
12		X	X	X	X
13		X	X	X	
14		X		X	X
18			X		

Side effects

To measure the AChE inhibition during the subchronic pretreatment with PHY or PHY/SCO blood samples were collected from the ear vein at three, seven and eleven days (day 3, 7 and 11) after osmotic mini-pump insertion. The effects on behavioural and neurophysiological parameters were tested during the first week of subchronic pre-treatment (see Table 1).

Protection against lethality induced by 2x LD_{50} soman

The efficacy of subchronic PHY or PHY/SCO-pretreatment with or without AS therapy in counteracting soman-induced lethality was investigated eleven days after implantation of the osmotic mini-pumps. The osmotic pumps were not removed. For the determination of AChE inhibition, blood samples were collected from the ear vein one and two hours after soman intoxication. The lethality was determined at 24 and 48 hours after soman intoxication.

Protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman:

The efficacy of subchronic PHY or PHY/SCO-pretreatment with or without AS therapy in counteracting soman-induced post intoxication incapacitation effects was investigated by observing the post intoxication symptoms like hyper-salivation, tremors and convulsions and by measuring behavioural and neurophysiological parameters. The observation of the symptoms started immediately after soman intoxication by investigators unaware of the treatment. After the intoxication symptoms became less severe the animals were able to perform in the behavioural and neurophysiological test systems. These tests started 2 hours after soman intoxication (day 11) and were repeated at different days (see Table 1).

Behavioural tests

1) *Shuttlebox performance*

In this test the active avoidance of an unpleasant event upon a conditioned stimulus (CS) is used to measure the retrieval of learned behaviour. For this test an automated two-way shuttlebox, consisting of two equal compartments of 23x23x23 cm with rounded corners, connected by a photo-cell-guarded gate, is used. The animals have 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 (CS). During the daily training and test sessions the animals receive 20 trials at an intertrial interval of 20-30 s (random). Only animals that reach the criterion of 80% or more correct avoidance reactions (CARs) after training, were used in the experiments (for details see Philippens *et al.*, 1992a). The number of CARs was used to express the active avoidance performance.

2) *Auditory startle response*

In this test the stretching movement of the hindpaws is used to measure the reaction of the animal on a startle signal (Davis *et al.*, 1982). For this test the animals are 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 200 ms duration is measured by a transducer connected with the platform, registering the force exerted by the animal upon presentation of the stimulus. An AD converter of an IBM-compatible PC digitised the responses. The area under the curve (AUC), amplitude and latency of the startle response are registered and used to express the motor reaction of the startle reflex.

Neurophysiological measurements

Under halothane/N_2O anaesthesia a silver electrode is placed 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 is placed over the nasal cavity. Both electrodes are connected with a plug and fixed on the skull with dental cement. During the test, the animals are immobilised in a vertically mounted PVC tube (as for the startle response) and a transmitter is connected to the plug for telemetric registration of the EEG and VER. All EEG signals were amplified (50.000x), filtered (between 0-30 Hz for EEG and 0-500 Hz for VER) and fed into an AD converter of an IBM-compatible PC; sampling frequency was 50 Hz for EEG and 1 kHz for VER.

1) EEG registrations

Fast Fourier transformation (FFT), to obtain power spectra, is performed from 5 randomly chosen EEG epochs of 12 s out of a total recording time of 5 min. The obtained power spectra of the guinea pigs are averaged per group and subdivided into 8 frequency classes (delta1: 0.8-2, delta2: 2-3.5, theta1: 3.5-5.5, theta2: 5.5-7.5, alpha1: 7.5-10, alpha2: 10-12.5, beta1: 12.5-18, beta2: 18-25 Hz). The total power (V^2) of the different frequency classes are used for the evaluation of the brain activity.

2) Visual Evoked Response (VER) measurements

For registration of the VER the animals receive 30 light stimuli with a time interval of 2 s ± 20%. Following the stimuli the EEGs are registered during 250 ms and the responses

averaged. For evaluation of effects the latencies and amplitudes of the positive (P1,P2,P3, P4) and negative (N1,N2,N3) peaks are measured and compared with the baseline values.

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 (Jonsons and Russell, 1975). 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.

Statistics

For statistical analysis of the behavioural tests an analysis of variance (ANOVA) was used and for the neurophysiological tests a paired t-test. For the survival and symptomatology after soman intoxication a Fisher exact probability test was used. In all tests p values < 0.05 were considered significant.

Results

In this study four different treatment regimens were tested on their efficacy against the toxic influences of 2x LD_{50} soman. Two groups received only a subchronic pretreatment with PHY (0.025 mg/kg/hr) alone (PHY) or PHY in combination with SCO (0.018 mg/kg/hr) (PHY/SCO) and two groups received also a post intoxication therapy with AS (17.4 mg/kg im) (PHY/AS or PHY/SCO/AS). First the occurrence of side effects after subchronically administered PHY (n=13) or PHY/SCO (n=12) was examined. Thereafter, two aspects were studied: protection against lethality induced by 2x LD_{50} soman, and protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman.

Side effects of subchronic administration of PHY or PHY/SCO

Body weight

All the animals of both pretreatment regimens (PHY, n=13 and PHY/SCO, n=12) gained weight amounting 40-60 grams during the pretreatment period of 11 days. This fits in the normal growth curve for guinea pigs in our laboratory.

Blood AChE inhibition:

The mean blood AChE-inhibition of the subchronic PHY-treated animals (n=13), measured as a percentage of their control value before osmotic pump implantation was: 20.3 ± 2.0 %; for the PHY/SCO-treated animals (n= 11) it was: 21.2 ± 5.2 %.

Side effects on behavioural parameters:

The measurements of possible behavioural and neurophysiological side effects were started three days after the implantation of the osmotic pumps containing PHY or PHY/SCO. In the shuttlebox task none of the two sessions showed any aberration on the performance (CAR) in both groups compared with their baseline value after training (PHY: 17.3 $\pm$0.63 and 16.4 $\pm$0.8 (baseline: 16.6 $\pm$0.56), PHY/SCO: 18.6 $\pm$0.5 and 18.4 $\pm$0.4 (baseline: 17.7 $\pm$0.7)). The startle

response measured at days 3 and 7 after pump insertion showed no aberrations compared with the baseline values. The amplitudes measured at day 7, expressed as a percentage of the baseline value, was in the PHY groups 119.7 ± 29, and in the PHY/SCO group it was 94.1 ± 18 (ANOVA: $p>0.05$).

Side effects on neurophysiological parameters:
The neurophysiological tests were performed 6 days after pump insertion.
The EEG activity was expressed as a power spectrum after FFT that was subdivided in 8 spectral bands. The total band power (V^2) in the different frequency classes of both groups (PHY or PHY/SCO) showed no difference compared with their baseline values.
The VER consists of four positive and three negative peaks. The amplitude and the latency of each peak were measured. The data were averaged per group and compared with their baseline value.
Neither the amplitudes nor the latencies changed in both groups (PHY or PHY/SCO) compared with their baseline values (t-test; $p>0.05$).

Protection against lethality induced by 2x LD_{50} soman

Body weight
The body weight of the animals in the groups treated with AS after soman intoxication was not affected when measured 24 hours after the intoxication. The animals in the PHY/SCO group showed significant loss in body weight (from 584 ± 17 g to 534 ± 11 g (n=5); $p=0.043$). The body weights of the animals from the PHY group could not be measured since they all deceased within 24 hours after soman intoxication.

Blood AChE inhibition
The mean blood AChE inhibition measured one and two hours after soman intoxication of the different treatment groups of animals compared to the control values before pump implantation are shown in Table 2. There are no significant differences in AChE inhibition in the different groups.

Table 2

Blood AChE inhibition after 2x LD_{50} soman intoxication in the different treated groups of animals (mean ± SEM, n= 6/group).

	AChE inhibition in % (± SEM)			
	PHY	PHY/SCO	PHY/SCO/AS	PHY/AS
1h after soman	87.0 (0.9)	88.5 (1.0)	87.2 (1.4)	85.6 (0.6)
2h after soman	90.9 (1.9)	91.2 (0.8)	89.0 (1.0)	89.6 (1.8)

Protective efficacy against soman induced lethality
All animals of the PHY/AS (n=7) and the PHY/SCO/AS (n=6) groups survived the intoxication with 2x LD_{50} soman completely and only one animal from the PHY/SCO group

(n=6) died already after 13 min. Of the PHY treated group all the animals died after soman intoxication. Five guinea pigs of this group deceased 1 hour and one guinea pig 4 hours after soman intoxication. Soman induced lethality was significantly higher in the latter group compared with the other treatment groups (Fisher exact probability test, $p<0.05$, two-tailed).

Protection against post-intoxication incapacitation after 2x LD_{50} soman intoxication

Post intoxication incapacitation symptoms

a) Subchronic PHY pretreatment and AS therapy against 2x LD_{50} soman

Most animals in this group only showed mild tremors, some ataxia and muscle fasciculations, lasting from 7 min after intoxication till about 1.5 h after intoxication. Three out of seven animals showed convulsive activity lasting for periods of about 4 min. No signs of dyspnoea were noticed. The day after soman intoxication all the animals suffered from hyper-salivation. This was not found in the groups of animals that received SCO in their pretreatment regime.

b) Subchronic PHY/SCO pretreatment and AS therapy against 2x LD_{50} soman

The animals of this group appeared to be in a better condition than the animals in the other three groups. The appearance of signs started about 10 min after soman intoxication. This was significantly later compared with the other groups (Fisher exact probability test, $p<0.05$, two-tailed). All animals showed mild to severe tremors. Only two animals suffered from convulsions (lasting 2 and 23 min). No signs of dyspnoea were noticed. The signs disappeared after 1.5 hours, and most animals were in a healthy condition.

c) Subchronic PHY pretreatment against 2x LD_{50} soman

Most animals (five out of six animals) showed severe tremors and convulsions lasting for periods of 7 to about 20 min. The other animal showed slight tremors and some ataxia followed by a period of dyspnoea. During the convulsive activity the animals started to suffer from dyspnoea that lasted until they died. All the animals in this group also showed effects on their eyes. Their eyes enlarged and "edematous" hydrolated. The first symptoms started within 5-10 minutes after intoxication. The clinical symptoms of the animals in this group were significantly worse compared with the groups treated with AS (Fisher exact probability test, $p<0.05$, two-tailed).

d) Subchronic PHY/SCO pretreatment against 2x LD_{50} soman

The animals of this group were in a much worse condition compared to those treated with AS after soman intoxication. Four out of six animals showed convulsions lasting for periods of 2, 3, 20, and 23 min., followed by a period of muscle fasciculations and dyspnoea. Only the animal that showed convulsions for 23 min did not suffer from dyspnoea, and the animal that suffered only 2 min from convulsive activity died very shortly after the soman intoxication. The remaining animals all suffered from severe tremors and dyspnoea. The signs started about 8 min after soman intoxication and lasted until 2.5 hours. The duration of the symptoms was significantly longer compared with the two groups that received AS therapy (Fisher exact probability test, $p<0.05$, two-tailed).

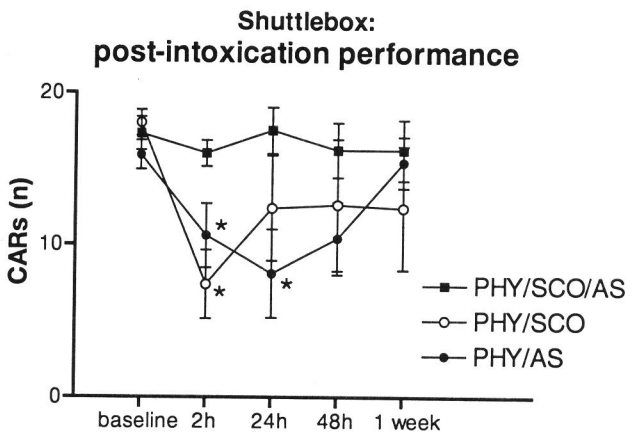

FIG.1: Post-intoxication effects on the shuttlebox performance before pump insertion (baseline) and 2, 24, 48 h and 1 week after soman (2x LD_{50} sc) intoxication (number correct avoidances, mean ± SEM). Three groups were tested: PHY/SCO (n=5), PHY/SCO/AS (n=6), and PHY/AS (n=7). The animals were pretreated with PHY (0.025 mg/kg/hr) alone or combined with SCO (0.018 mg/kg/hr). Two groups received a treatment with AS (17.4 mg/kg im) one min after soman intoxication. *significant compared with the baseline value ($p<0.05$).

Post-intoxication incapacitation effects on behavioural measurements

The post-intoxication incapacitation effects on the shuttlebox performance after soman intoxication are shown in Fig. 1. All the animals that survived the intoxication were able to perform the task in the shuttlebox; they showed a normal inter-trial response (ITR) activity (compartment changes during the inter-trial interval). The animals from the PHY/SCO/AS group performed very well in this task, whereas the animals from the PHY/AS, like those of the PHY/SCO group, showed a significant decrease of their CARs (an ANOVA analysis at four hours after soman showed p=0.042 and p=0.006 respectively and at 24 hours after soman p=0.033 for the PHY/AS group). One week later the performance of the animals in the PHY/AS group returned to normal baseline value. At that moment the performance of the PHY/SCO group was still affected (not significant). This was mainly due to the fact that the animals that performed very bad in this task shortly after soman did not recover during that week, whereas the animals that only showed a mild decrease of the performance recovered almost completely after one week.

The effects on the startle response observed after 2x LD_{50} soman intoxication are shown in Fig. 2. In all the groups an increase of the startle response was observed. The results indicate that a single dose of AS enhanced the effect on the startle reflex after soman intoxication in PHY or PHY/SCO pretreated animals, whereas the addition of subchronical administered SCO increased the duration of the effect.

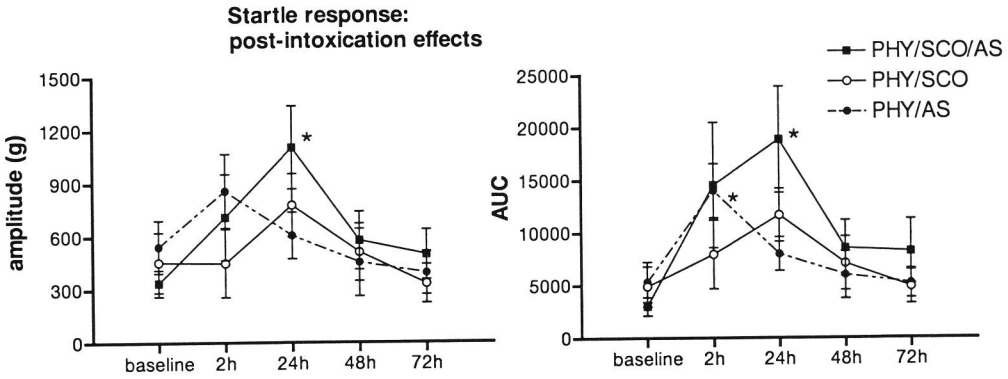

FIG. 2: Post-intoxication effects on the startle response amplitude and AUC before pump insertion (baseline) and 2, 24, 48, and 72 hours after soman (2x LD_{50} sc) intoxication (mean ± SEM). Three groups were tested: PHY/SCO (n=5), PHY/SCO/AS (n=6), and PHY/AS (n=7). The animals were pretreated with PHY (0.025 mg/kg/hr) alone or combined with SCO (0.018 mg/kg/hr). Two groups received a treatment with AS (17.4 mg/kg im) one minute after soman intoxication. *significant compared with the baseline value ($p<0.05$).

The animals from the PHY/AS group showed an increase of the startle response shortly after soman intoxication. This increase affected the AUC significantly (p= 0.02) at 2 hours after soman intoxication. Furthermore, at this time point also the latency of the response was significantly delayed (p= 0.013). The animals from the PHY/SCO/AS group showed a significant increase of the startle response measured at 24 hours after soman intoxication (amplitude: p= 0.021, AUC: p= 0.025). The effects on the startle response in the PHY/SCO group were not found to be significant.
Two days after soman intoxication the startle responses of all the groups were back to their baseline values. The animals of the PHY group could not be measured becaused they died before the start of the test. Only one animal of the PHY group that deceased 4 hours after soman was tested in the startle response task. This animal showed a very weak response.

Post-intoxication incapacitation effects on neurophysiological measurements
The EEGs were measured 2, 24, and 72 hours after soman intoxication (data not shown). In the PHY/SCO/AS group a significant decrease of the power was found in the alpha2 and beta1 bands 2 hours after the intoxication (p=0.0001 and p=0.024). In the PHY/SCO group a significant increase of the power was found only in the alpha2 band 24 hours after the intoxication (p=0.0003) and in PHY/AS group in the alpha1 band 72 hours after the soman intoxication (p=0.027). No effects were found in the delta and theta bands.

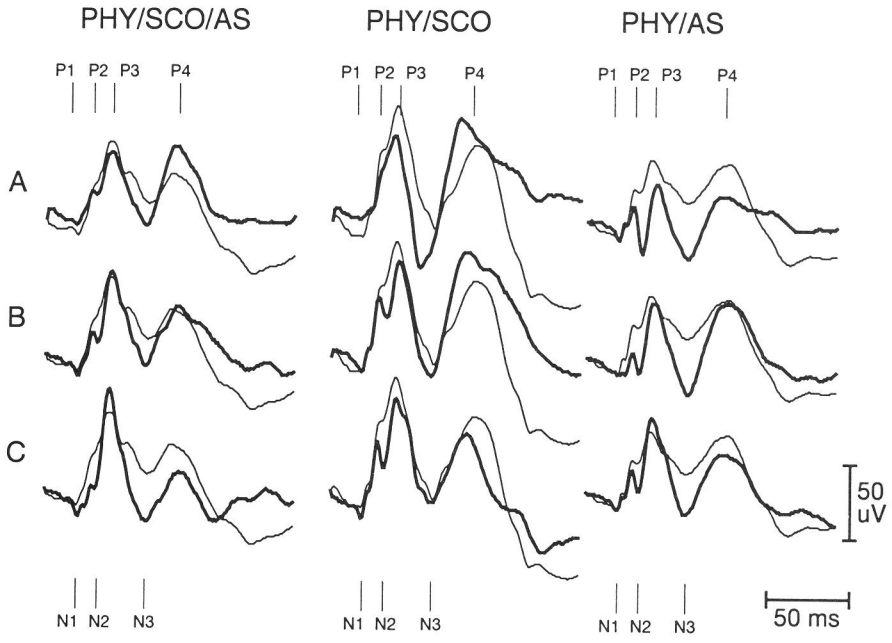

FIG. 3: Post-intoxication effects on the VER (thick lines) compared with the baseline values (before pump insertion, thin lines) registered at A: 2, B: 24, and C: 72 hours after soman (2x LD_{50} SC) intoxication. Three groups were tested: PHY/SCO/AS (n=6), PHY/SCO (n=5), and PHY/AS (n=7). All animals were pretreated with PHY (0.025 mg/kg/hr) alone or combined with SCO (0.018 mg/kg/hr). Two groups received a treatment with AS (17.4 mg/kg IM) one minute after soman intoxication. The averaged VER curves from equally treated guinea pigs are shown in which the four positive peak (P1, P2, P3 ,P4) and three negative peaks (N1, N2, N3) are indicated.

The VERs of the different treatment groups measured at 2, 24, and 72 hours after soman are shown in Figure 3. The peak amplitudes and latencies of each animal were measured, averaged per treatment group and compared with their own control values. For all test points in the different groups no effect was found on the latency of the VER peaks after 2x LD_{50} soman intoxication (at all registration points $p>0.05$). The amplitudes of the responses on the other hand, seem to be affected after soman exposure. An increase of the N3 peak was found in all the groups at 2 hours after the soman intoxication and in the AS treated groups at all registration points. This was found to be worst in the PHY/AS group but not to be significant because of the variation between the animals. Furthermore, a slight but not significant increase was found of the N2 peak especially in the PHY/AS group (at all registration points $p>0.05$).

Discussion

In this study the efficacy of the addition of SCO to the subchronic PHY pretreatment in a therapeutically relevant dose (Gall *et al.*, 1981), against 2x LD_{50} soman was tested. This was done by comparing both pretreatment regimens in the absence or presence of a post intoxication therapy with AS. Two aspects were studied: the protection against lethality induced by 2x LD_{50} soman, and the protection against post-intoxication incapacitation after intoxication by 2x LD_{50} soman.

Before the efficacy of the different treatment regimens was examined the pretreatment with PHY or PHY/SCO was tested for the occurrence of behavioural and neurophysiological side effects. In the tests used, no behavioural or neurophysiological side effects were found during subchronic pretreatment with PHY or the combination of PHY and SCO (18 µg/kg/hr). These findings confirm the results found in an earlier study with guinea pigs (Philippens *et al.*, 1998). In that study almost the same protocol was followed for testing side effects of PHY or PHY/SCO pretreatment.

Protection against lethality induced by 2x LD_{50} soman

The use of a post-intoxication therapy with AS enhanced the protection against soman induced lethality. Animals that received AS immediately after soman intoxication survived completely, whereas the PHY pretreated animals that did not receive a post-intoxication therapy were not protected against the toxicant. They all deceased about one hour after soman. It has been reported in the literature that guinea pigs only pretreated with acute PHY all died within 30 minutes of 2xLD_{50} soman administration (Lim *et al.*, 1988b). When a single dose of AS was given after soman-intoxication the protective ratio of a carbamate, like PYR or PHY, was considerable improved (Gordon *et al.*, 1978; Leadbeater *et al.*, 1985; Meshulam *et al.*, 1995). This is noteworthy, since the protective ratio of AS alone has been reported to be 1.5 (Inns and Leadbeater, 1983).

When the PHY pretreatment was combined with SCO the animals were able to withstand the soman intoxication; only one animal died. It appeared that the blood AChE of this animal was not inhibited during the pretreatment period. Presumably the osmotic pump had not delivered PHY + SCO properly, resulting in insufficient pretreatment. Indeed the survival time after soman intoxication of this animal was as short (13 min) as in untreated animals. A higher protection against soman induced lethality by addition of SCO to the PHY pretreatment has been reported in literature (Leadbeater *et al.*, 1985; Lim *et al.*, 1988b; Meshulam *et al.*, 1995). Remarkably, SCO alone did not offer any protection against soman induced lethality (Wetherell, 1994). It can be concluded that addition of SCO to PHY as pretreatment or addition of AS as post intoxication therapy enhance the protection synergistically against soman induced lethality. In case the post-intoxication therapy is not used, the addition of SCO in the pretreatment regime is of life saving importance.

Protection against post-intoxication incapacitation after 2x LD_{50} soman

The treatment regime consisting of a pretreatment with PHY and SCO followed by a post-intoxication therapy with AS (PHY/SCO/AS) offers the best protection against the soman induced intoxication symptoms. In those cases where no therapy with AS was administered the addition of SCO improved the protecting effect against soman induced symptoms. This

improvement has also been reported by others (Leadbeater *et al.*, 1985; Lim *et al.*,1988b; Wetherell, 1994). SCO even offers a better result in counteracting soman or sarin induced incapacitation than AS post intoxication therapy given to guinea pigs that were pretreated with a single dose of PHY (Leadbeater *et al.*, 1985). The positive effects in reducing post intoxication incapacitation of the addition of cholinolytics to PHY are also reflected in the shuttlebox data. A correlation is observed between the degree of intoxication symptoms and the performance in the shuttlebox. Such a correlation was described earlier in rats that only received therapy after soman intoxication (Philippens *et al.*, 1992b). On the other hand, in the startle response task another picture appears. In all the situations an increase or a tendency towards an increase of the startle response was found. The addition of a single dose of AS significantly enhanced the effect on the startle reflex after soman intoxication in PHY or PHY/SCO pretreated animals. In a previous study it was clarified that direct effects on nicotinergic receptors were involved in the effects on the startle amplitude instead of AChE inhibition (Philippens *et al.*, 1997). In that study a single dose of the muscarinic antagonist SCO antagonised the decreasing part of the bellshape dose response curve of PHY on the startle reflex, leading to an increase of the response. This part of the curve could be mimicked by soman. Therefore, the increase of the startle response after a single injection with AS in pre-treated and soman intoxicated guinea pigs may be a result of the antagonising effect on the decrease of the startle response. For this reason, the animals of the PHY/SCO group seemed to be less affected than those of the PHY/SCO/AS group. Presumably, this strong effect on the startle amplitude may be less when the very high dose of AS is lowered compared with the realistic situation in human (2 mg/70 kg). Furthermore, PHY administered in a single dose causing an increase of the startle amplitude did also affect the VER (Philippens *et al.*, 1997). This effect on the N3 peak could, like the effect on the startle, not be counteracted by SCO. Therefore, these effects are most likely not the result of AChE inhibition. In this study a comparable tendency was found on the VER in all the treatment groups. These findings strengthen the idea that other factors, like direct effects on nicotinergic receptors, play a role in the post intoxication incapacitation after soman. Interestingly, not only PHY exerts agonistic effects on nicotinergic receptors (Albuquerque *et al.*, 1984; Sherby *et al.*, 1984) also soman seems to exert such effects (Bakrey *et al.*, 1988). This may influence the protecting efficacy against soman intoxication.

From the present experiments it may be concluded that the use of SCO as an adjunct pretreatment drug is not necessary to depress possible side effects provided PHY pretreatment is given subchronically. However, in case soldiers are unable to use or are deprived from a post-intoxication therapy the addition of SCO to the PHY pretreatment can be life saving. The pretreatment regimen of PHY + SCO offers sufficient protection against lethality but did not improve the post-intoxication incapacitation when compared with a post-intoxication therapy with AS after PHY or PHY + SCO pretreatment. These post-intoxication effects could not completely be explained purely by AChE inhibition. Presumably, direct effects on nicotinergic receptors are also involved. In support of this opinion is the observation that after subchronic treatment with OPs muscarinergic and nicotinergic receptors become subsensitive to acetylcholine (Costa and Murphy, 1983; Bhat *et al.* 1990). Therefore, the treatment may further be improved by the addition of a nicotinergic antagonist.

In conclusion, subchronic treatment with the combination of PHY and SCO seems to be a better alternative for the current PYR pretreatment than PHY alone since it improves the protection against soman induced lethality in case a post-intoxication therapy is not available. To further improve the efficacy of the treatment regime against post-intoxication incapacitation, the addition of a nicotinic antagonist would be advisable. Such a treatment scenario will be examined in the future.

8

General discussion

As outlined in the introduction, the current pretreatment against OP intoxication with pyridostigmine is far from ideal. Hence, a more effective sign-free pretreatment is needed. It has been proposed to replace pyridostigmine by the tertiary carbamate physostigmine (Leadbeater *et al.*, 1985). This centrally effective compound has been shown to be effective against OP intoxication (Gordon *et al.*, 1978; Leadbeater *et al.*, 1985; Miller *et al.*, 1993; Wetherell, 1994). In this thesis research efforts are described to examine the potential of physostigmine as a pretreatment against OP intoxication.

The prerequisites for a successful pretreatment

Before physostigmine may replace pyridostigmine as a pretreatment against OP intoxications some questions should to be clarified. One of these questions is, does physostigmine cope with the three conditions of a successful pretreatment, i.e.: 1) offering a high protection rate against lethality, 2) causing no side effects, and 3) offering protection against post intoxication incapacitation? Physostigmine has been tested with respect to these prerequisites.

1) Protection against lethality

To examine the protective efficacy of physostigmine pretreatment against OP induced lethality physostigmine was administered prior to soman intoxication. In the Chapters 5 and 6 a high protective efficacy against soman induced intoxication is reported in both guinea pigs and marmoset monkeys. Guinea pigs that were pretreated with a single dose of physostigmine in combination with scopolamine and atropinised one minute after intoxication with $3xLD_{50}$ soman all survived (Chapter 5). Whereas only 43% of the guinea pigs pretreated with a comparable dose of pyridostigmine (0.04 mg/kg sc) in combination with scopolamine survived. These findings suggest that physostigmine is a more effective pretreatment than pyridostigmine. This is in accordance with earlier reported findings (Solana *et al.*, 1990; Miller *et al.*, 1993). These investigators compared the efficacy of pyridostigmine and physostigmine pretreatment against soman intoxication in guinea pigs and rats respectively and found a better protective effect against lethality after physostigmine pretreatment. The protective efficacy of pyridostigmine reported in Chapter 5 was worse compared with the one reported by Gordon *et al.*(1978). They found a protective ratio of 8.0 with pyridostigmine (0.10 mg/kg im) against soman intoxication. Since these investigators applied pyridostigmine im instead of sc different pharmacokinetics may explain the difference in protective efficacy. Such a difference was also found for the phosphoramidate PNF. When this cholinesterase (ChE) inhibitor was administered iv 30 minutes prior to soman intoxication a high protective ratio was found (Langenberg *et al.*, 1996), whereas a sc injection with the same pre-dose time leading to a comparable AChE-inhibition did not protect against soman induced lethality (Melchers *et al.*, 1994). This suggests that for effective protection against lethality the route of administration is important, most likely because these different routes of administration may lead to different carbamate or phosphoramidate plasma levels. A chronic transdermal application of physostigmine offering a ChE inhibition of 53 % leads to a plasma level of 4.1 $\pm$ 0.8 ng/ml, while a single im injection that offers almost the same ChE inhibition of 59 % leads to a plasma level of 14 $\pm$ 1.3 ng/ml (Meshulam *et al.*, 1995). Therefore, single iv injection will cause a high plasma level of the compound immediately after injection. Such an accumulation of the carbamate in the circulation will direct the carbamoylation reaction to the

right (see Fig. 3 of the Introduction) leading to a high rate coefficient for decarbamoylation of carbamoylated enzyme (Watts and Wilkinson, 1977). When a slow release strategy is followed the reaction will reach a steady state: the rate coefficient for carbamoylation of the enzyme from the enzyme/carbamate complex will be almost equal to that for decarbamoylation of carbamoylated enzyme. This situation is aimed by the current pretreatment that consists of repeated oral pyridostigmine administration. From the forementioned it may be clear that the protection against lethality obtained by a single injection of pyridostigmine or physostigmine can not predict the efficacy that will be obtained after continuous administration. For that reason the efficacy against lethality of a more chronic application should be considered and experimentally evaluated. In Chapter 5 physostigmine subchronically administered by an osmotic mini pump at a therapeutically relevant dose, alone or in combination with scopolamine, was tested for its efficacy in guinea pigs. These animals were, like those that were pretreated with a single dose of physostigmine, protected against a 3x LD_{50} soman intoxication. This finding corroborates an earlier report that described a similar protection of acute and subchronic physostigmine, both combined with scopolamine, against the toxic influences of 2 or 5x LD_{50} soman in guinea pigs (Anderson *et al.*, 1991). Therefore, physostigmine seems to fulfil the first condition of a successful pretreatment. To increase the probability that extrapolation of these data to man is allowed, this procedure was also examined in the marmoset monkey (Chapter 6). After subchronically administered physostigmine, without the addition of scopolamine, marmoset monkeys were completely protected against lethality induced by 2x LD_{50} soman (sc) intoxication, provided a therapy with atropine sulphate was given.

2) Causing no side effects

It may be clear that drugs when given prophylactically to healthy persons should be devoid of side effects. In particular when these drugs have to be taken for several weeks or even months. Since physostigmine also inhibits AChE in the brain, the accumulation of ACh could lead to unwanted central side effects. Most of these side effects can be counteracted by a cholinolytic drug. In most of the published studies a cholinolyticum was added to the pretreatment to acquire a better protection against OP intoxication (Leadbeater *et al.*, 1985; Lim *et al.*, 1988b; Meshulam *et al.*, 1995). A cholinolyticum is also one of the drugs applied as post intoxication therapy to prevent further overstimulation of the cholinergic receptors. In the studies described in this thesis the centrally active cholinolyticum scopolamine is added to the pretreatment regime to prevent physostigmine-induced side effects (Chapter 3). The high penetration into the brain makes this compound well suited to counteract the side effects of physostigmine. Indeed, scopolamine is better in antagonising the physostigmine-induced behavioural suppression than atropine (Genoves *et al.*, 1990). This antagonising capacity of scopolamine is described in Chapter 3: scopolamine was shown to prevent unwanted behavioural and neurophysiological side effects. Not all the side effects caused by a single dose of physostigmine could be prevented by scopolamine. It could be demonstrated that some effects were due to a direct action of physostigmine on nicotinergic receptors (Chapter 4). Interestingly, nerve agents and in particularly VX and soman also affect ACh receptors (Bakry *et al.*, 1988). Bakry et al. reported that soman at micromolar concentrations can act as a partial agonist of the nicotinergic ACh receptor and can induce receptor desensitisation. At

lower concentrations VX and soman may also affect a small population of muscarinergic receptors that show the same affinity for the OP compound as AChE. This observation suggested that the toxicity of soman might be due to a combined action on nicotinergic and muscarinergic receptors. The direct effect of physostigmine on nicotinergic receptors can therefore be of importance for the protecting efficacy of physostigmine.
Fortunately, no side effects were observed during subchronic physostigmine treatment, even without scopolamine (Chapter 5). One possible explanation could be the development of tolerance against AChE inhibitors. However, the absence of side effects was also observed in the startle reflex: these results were considered not to be related to AChE inhibition (Chapter 4). Presumably, adaptation also occurred on nicotinergic receptors sites, since soman may act on nicotinergic and muscarinergic receptors, thereby amplifying the protecting efficacy of physostigmine.
The finding that a more practical administration of physostigmine by subchronic application did not lead to side effects suggests the use of a cholinolyticum to be redundant. This is corroborated by the results observed in the study where marmoset monkeys were used instead of guinea pigs (Chapter 6). In the primate the subchronic administration of physostigmine alone, at a dose leading to an AChE inhibition of 30 %, did not lead to physical, behavioural and neurophysiological side effects.
Concerning the prevention of unwanted side effects one can conclude that a subchronic application of physostigmine offers the best results. The addition of scopolamine as a supplement to the pre-treatment regime may not be necessary.

3) Protection against post intoxication incapacitation.

A successful pretreatment should not only protect against lethality but also against the incapacitation due to the OP intoxication. Most of these incapacitating effects are caused by the high AChE inhibition and are manifested by muscarinic and nicotinic peripheral and central signs.

Intoxication symptoms

After OP intoxication, at first characteristic signs and symptoms of intoxication will appear. These symptoms have mostly been used in studies to determine incapacitation effects (Wetherell, 1994; Miller *et al.*, 1993; Solana *et al.*, 1990). These symptoms can offer a good indication of how effective a pretreatment is in reducing post intoxication incapacitation. Through comparison of different pretreatment regimes it was already shown in rats that a subchronic pretreatment with physostigmine protected significantly better against soman induced incapacitation than pyridostigmine (Miller *et al.*, 1993). In Chapter 5 different applications and combinations of physostigmine pretreatment were tested and compared in counteracting soman-induced symptoms in atropinized guinea pigs. The order of protective efficacy was: acute physostigmine+scopolamine > subchronic physostigmine > subchronic physostigmine+scopolamine. The better protective effect of the acute pretreatment can be explained by the higher AChE inhibition, since a relation has been reported between AChE inhibition and protecting efficacy against OP intoxication (Harris *et al.*, 1989). In case of the sign free subchronic pretreatment regimes, the treatment with physostigmine alone seemed to offer the best protection against 3x LD_{50} soman induced symptoms (Chapter 5). A clear

protection of subchronic physostigmine was also observed after 2x LD_{50} soman in marmoset monkeys (Chapter 6). However, guinea pigs that had received a pretreatment with physostigmine and scopolamine appeared to recover faster (Chapter 7). This was also confirmed by other investigators: Guinea pigs pretreated with subchronic physostigmine showed no obvious signs of poisoning at 4 h post-intoxication, whereas in the guinea pigs pretreated with the combination of subchronic physostigmine and scopolamine the signs already had disappeared 2 h post-intoxication (Wetherell, 1994).

Behavioural and neurophysiological post-intoxication effects

When the first signs of intoxication have disappeared the animals may still suffer from the OP intoxication. This is most accurately reflected by behavioural and neurophysiological parameters. Because physostigmine easily penetrates the brain it should protect the CNS against toxic influences of OPs. Indeed, the protective ratio in guinea pigs against post soman intoxication incapacitation, measured by gross motor performance in a swimming test, was found to be 2.0 after physostigmine pre-treatment and 1.0 after pyridostigmine pre-treatment (both at a comparable dose leading to a peak erythrocyte AChE inhibition of 70%) (Leadbeater *et al.*, 1985).

In this thesis not only the clinical signs but also the behavioural performance in different test systems and the spontaneous and evoked brain activity were measured to study post-intoxication incapacitation.

This was examined after most severe symptoms of soman (2x LD_{50}) intoxication had disappeared (Chapter 7). The shuttlebox performance was not affected in the guinea pigs that were pretreated with subchronic physostigmine and scopolamine, whereas, the guinea pigs that received only subchronic physostigmine pretreatment showed a decline in their performance and were not able to recover in this task within a week. On the other hand this latter group responded normal again in the startle response task 24 hours after soman, while in the physostigmine/scopolamine/atropine group it took 24 hours longer. Similar post soman intoxication effects were observed in subchronic physostigmine pre-treated marmoset monkeys in the startle reflex (Chapter 6). These animals performed almost normal in all the tasks shortly after soman intoxication.

To prevent or reduce the post intoxication incapacitation, one can conclude that the subchronic pre-treatment with physostigmine or physostigmine and scopolamine offer both a high reduction of the incapacitation after 2xLD_{50} soman in case a post intoxication therapy with atropine is used.

What should be the ideal regime for physostigmine pretreatment with respect to OP intoxication?

Concerning the prerequisites of a successful pretreatment the subchronic pretreatment with physostigmine alone seems to be a better alternative for the current pretreatment with pyridostigmine. This was established in experiments in which the laboratory animals received a post-intoxication therapy with atropine after soman intoxication. However, one can imagine situations that soldiers are deprived of or are not capable of using an auto-injector containing therapy. It was reported that a pretreatment with a carbamate alone did not offer any protection against lethality (Lim *et al.*, 1988b; Gordon *et al.*, 1978). This was confirmed in this thesis: all the animals pretreated subchronically with physostigmine alone died; the animals that received a post intoxication therapy with atropine survived after 2x LD_{50} soman intoxication (Chapter 7). Furthermore, animals that received the complete pretreatment (subchronic physostigmine/scopolamine) without a post-intoxication therapy also survived. In this situation the addition of scopolamine in the pretreatment regime is of vital importance. Interestingly, the treatment with a cholinolytic alone did not protect against soman induced lethality. Atropine alone has a protective ratio of only 1.5 against soman intoxication (Inns and Leadbeater, 1983), and scopolamine alone did not offer any protection (Wetherell, 1994).
A pretreatment regime that fully protects against lethality and post intoxication incapacitation not only induced by soman but also by many of the known nerve gases would solve the problems that still consist in the oxime therapy. This was stated by Wolthuis *et al.* in 1981 but is still valid. Therefore, the aim of the pretreatment should be to abolish all the effects of the toxicant.
So far it can be concluded that subchronic pretreatment with physostigmine and scopolamine seems to be the best choice to prevent OP intoxication, although some post intoxication effects on the startle reflex still exist (Chapter 7). These effects may be related to direct effects on nicotinergic receptors. In that case, the addition of a nicotinergic antagonist would be advisable to further improve the efficacy of the pretreatment regime.

TABLE 1

Overview of the data obtained with different pretreatment regimes: appearance of side effects during pretreatment and protection against soman intoxication followed by atropine therapy[a].

Pre-treatment	AChE activity	Side effect	soman xLD_{50}	Mortality	Intoxication symptoms		Intoxication incapacitation
					Convulsions	Dyspnoea	
Guinea pig							
Acute PYR	24.8	NT	3	4/7	4/7	5/7	NT
			1.5	0/8	0/8	0/8	>5
Acute PHY	44.5	++	3	0/6	1/6	0/6	NT
			1.5	0/8	0/8	0/8	<1
Acute PHY/SCO	50.5	+	3	0/8	1/8	1/8	NT
Subchr. PHY/SCO	33.3	-	3	2/8	7/8	5/8	NT
	21.2		2	0/6	2/6	0/6	<1
			2[b]	1/6	4/6	5/6	>7
Subchr. PHY	35.8	-	3	0/5	2/5	1/5	NT
	20.3		2	0/7	3/7	0/7	7
			2[b]	6/6	5/6	6/6	NT
Marmoset monkey							
Subchr. PHY	30.7	-	2	0/6	0/6	0/6	7
Control	0	-	2	3/3	3/3	3/3	NT

[a]Atropine therapy was given im one minute after soman (sc) intoxication (guinea pigs: 17.4 mg/kg , marmoset monkeys 5 mg/kg).

[b]No post-intoxication therapy with atropine was given.

The degree of side effects during pretreatment is expressed as follows: -: not observed, +: observed in 50% of the used read-out systems, ++: observed in all read-out systems. The mortality and intoxication symptoms are expressed as the number of animals per treatment group in which these effects occur. The post intoxication incapacitation was expressed in the maximal number of days after intoxication that effects were detectable in behaviour.

PHY: physostigmine, PYR: pyridostigmine, SCO: scopolamine, NT: not tested. (Data about pyridostigmine were collected from an internal report by Philippens, 1993)

How predictive are experimental data in guinea pigs and marmoset monkeys for pretreatment protocols to be applied in man?

One of the fundamental questions that arise from this study is: how predictive are the results obtained in guinea pigs and marmosets for man? Obviously, it is impossible to test the efficacy of carbamate pretreatment against a soman challenge in human volunteers. Therefore, studies with experimental animals are inevitable. Knowing that behaviour is regulated by the nervous system, effects of chemicals on the function of the nervous system, e.g., blockade of receptors, release of transmitters, etc., will probably affect behaviour as the consequence of the disturbed systems. In case of OP intoxication some examples are present in humans. By observing the symptomatology in man, animal models can be created that develop comparable signs, although some symptoms like hallucinations or headache is difficult to detect in animal models. On the other hand, behavioural models measuring deficits in motor, sensory and cognitive functions reveal qualitative effects that have some predictive value when compared with similar effects in humans. However, it is difficult to predict which functional domain in the brain is affected. Therefore, development of a variety of read-out systems is needed (Chapter 2). Still the question exists whether the data obtained from the different read-out systems for the guinea pigs and the marmoset monkeys are predictive for man. Therefore, an approach is needed to extrapolate from experimental data in the guinea pig and the marmoset to man. As stated before, the marmoset has been shown to be a suitable model for man in OP toxicity studies (Van Helden *et al.*, 1983). Likewise, from a toxicokinetic point of view the guinea pig appeared to be a better model for the marmoset than the rat (Benschop and De Jong, 1991). However, since marmosets or guinea pigs are not miniature humans, extrapolation on the basis of body weight, metabolic rate or ventilation rate will not be adequate. The most suitable approach for interspecies extrapolation is via physiologically based pharmacokinetic modeling (PBPK) (Dedrick *et al.*, 1973; King *et al.*, 1983; Ramsey and Andersen, 1984; Lutz *et al.*, 1984), albeit that in the case of soman one should replace 'pharmacokinetic' with 'toxicokinetic'. PBPK models represent the mammalian system in terms of specific tissues or groups of tissues, connected by arterial and venous blood flow pathways. They consist of a set of differential equations describing the mass-balance in the various tissues and groups of tissues. Based on these differential equations, time-dependent toxicokinetic data can be simulated. The model contains physiological parameters, such as tissue volumes and blood flow rates, and parameters specific for the chemical agent under investigation, such as tissue/blood partition coefficients and metabolic parameters. The coherent relationship among anatomical and physiological characteristics of different species provides the basis for cross-species scaling of toxicokinetic data described in such a model and extrapolation eventually to man. Nowadays, PBPK models are used extensively for risk assessment purposes. The results of this type of modeling are accepted by regulatory organizations.

Recently, Langenberg *et al.* (1997) have reported a PBPK model for the intravenous toxicokinetics of soman in the atropinized guinea pig. This model was validated by comparing the predictions of the model with the actual toxicokinetic data, i.e., measured time courses of arterial blood levels of soman following iv bolus administration of doses of soman corresponding with 0.8, 2 and 6 x LD_{50}. The model also contains differential equations for simulation of carbamate pretreatment. Furthermore, AChE activities can be predicted at any point in time during the simulation in all compartments defined in the model. Presently, the

physiologically based model developed by dr. J. P. Langenberg (PML-TNO) for the intravenous toxicokinetics of soman in the atropinised guinea pig is being adapted to allow modeling of nose-only exposure. Concomitantly, a model for the marmoset is under development. When these models are adequately validated, a model for the toxicokinetics of soman in man will be constructed. Until these models have been fully validated it remains uncertain whether our findings in the guinea pig and marmoset monkey can be directly extrapolated to man. It seems however at this point not unreasonable to assume that the sign-free subchronic pretreatment with physostigmine and scopolamine offers an effective protection against 2x LD_{50} soman induced lethality and post-intoxication incapacitation.

Which questions need to be clarified in the near future?

All the experiments in this thesis were performed in a laboratory situation. However, in a more realistic situation other factors may interfere with the pretreatment regimen.
After the Persian Gulf war soldiers were suffering from symptoms called the Gulf War Syndrome. Most symptoms were hard to determine and could be due to interaction with other drugs. These drugs can be nicotine or other psychoactive drugs. Some symptoms could be the result of the stress situation in wartime. It is known that stress can change the kinetics of the pretreatment (Friedman, 1996) and, therefore, affect the protective ratio. Therefore, the protective efficacy of physostigmine should also be examined in combination with other drugs or vaccines in a stress-full situation. Such circumstances may evoke the appearance of unwanted side effects and decrease protection against intoxication.
Furthermore, in this thesis the protection against OP intoxication was tested by using soman. The reason for choosing soman was its severe reactivity and high penetration degree into the CNS due to its lipophilic structure. However, a successful pretreatment should also protect against the toxic influences of other chemical nerve agents. Presumably this will be the case, since it has been reported that physostigmine protects against other OPs like VX and sarin (Leadbeater *et al.*, 1985; Anderson *et al.*, 1991). Finally to apply this pretreatment regimen in humans, a more practical route of administration should be followed than an osmotic mini-pump. The transdermal application via a plaster pad will be considered.

In conclusion, the results so far (see Table 1) demonstrate that subchronic application of physostigmine in combination with scopolamine seems to be a promising alternative to replace the current pretreatment with pyridostigmine against OP intoxication. This sign-free pretreatment offers, without additional therapy, the best protection against 2x LD_{50} soman in guinea pigs.

Nederlandstalige samenvatting

De toxische werking van organofosfaten berust op de remming van het enzym acetylcholinesterase (AChE) dat betrokken is bij de afbraak van de neurotransmitter acetylcholine (ACh) in cholinerge zenuwuiteinden. Door de remming van AChE zal ACh zich ophopen in de synaps-spleet. Dit kan leiden tot over-stimulatie van de receptoren op het effector orgaan en uiteindelijk resulteren in "verlamming" van het systeem. Incapacitering, blijvende hersenschade of in het ergste geval de dood zijn het gevolg. Door een deel van het enzym "af te schermen" tegen de inwerking van organofosfaten (OPs) kan men redelijk beschermd worden tegen zowel de incapaciterende als de letale effecten van deze verbindingen. Merkwaardigerwijs kan deze bescherming worden geleverd door een andere AChE-remmer, een carbamaat. Deze stof komt door middel van spontane hydrolyse gemakkelijk weer vrij van het enzym waardoor het enzym opnieuw geactiveerd wordt. Gewoonlijk worden deze stoffen reversibele AChE remmers genoemd. In veel NAVO-landen wordt momenteel als voorbehandeling het carbamaat pyridostigmine gebruikt. Deze voorbehandeling geeft een redelijke bescherming tegen de letale effecten van OP-verbindingen. Pyridostigmine dringt echter niet goed door in de hersenen waardoor deze niet goed beschermd worden tegen de schadelijke werking van OPs. Er is voorgesteld de huidige voorbehandeling te vervangen door een voorbehandeling met fysostigmine. Deze sterk aan pyridostigmine verwante verbinding is ook een reversibele remmer van het AChE maar kan, in tegenstelling tot pyridostigmine, wel in de hersenen doordringen. Hierdoor kan fysostigmine het AChE in de hersenen beschermen tegen binding met het zenuwgas en zo convulsieve activiteit en de daaruit voortvloeiende hersenschade voorkomen. Echter, een mogelijk probleem van de voorbehandeling met fysostigmine is dat het op zichzelf aanleiding kan geven tot ongewenste bijwerkingen, juist doordat het in de hersenen kan doordringen. Het blijkt dat fysostigmine, toegediend in een **eenmalige** therapeutische dosering in de cavia inderdaad aanleiding geeft tot een aantal bijwerkingen. Deze bijwerkingen kunnen, vertaald naar de humane situatie, de taakverrichting van de militair ernstig verstoren. De meeste van deze bijwerkingen, onder andere op aangeleerd gedrag, konden worden voorkomen door gelijktijdig met de toediening van fysostigmine een lage dosering van het cholinolyticum scopolamine toe te dienen. Echter, op andere parameters die werden verstoord door fysostigmine had scopolamine geen of juist een versterkend effect.

In de praktijk situatie zal een voorbehandeling met fysostigmine gedurende enige dagen tot weken moeten worden toegediend. Daarom zijn in dit proefschrift ook de effecten onderzocht van een subchronische toediening van fysostigmine, al dan niet gecombineerd met scopolamine. Het bleek dat zowel de subchronische behandeling met de combinatie van fysostigmine en scopolamine als die van fysostigmine alleen in de cavia geen aanleiding gaven tot gedrags- of neurofysiologische afwijkingen. Subchronisch toegediend fysostigmine induceerde ook in de marmoset aap geen bijwerkingen. De toevoeging van scopolamine aan de voorbehandeling bleek niet nodig te zijn om eventuele bijwerkingen te voorkomen. Tevens bleek de therapeutische effectiviteit van subchronisch toegediend fysostigmine tegen soman (gegeven in combinatie met een post intoxicatie therapie met atropine) erg hoog zowel in de cavia als in de marmoset aap. Echter, in het geval dat de militair, door welke omstandigheid dan ook, niet in staat zou zijn zichzelf de post intoxicatie therapie toe te dienen, blijkt de toevoeging van scopolamine de overlevingskansen aanzienlijk te vergroten. Bovendien wordt de post-intoxicatie incapacitering geminimaliseerd. Men kan concluderen uit deze experimenten dat subchronisch toegediend fysostigmine in combinatie met scopolamine een goed alternatief lijkt te zijn voor de huidige voorbehandeling met pyridostigmine.

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Curriculum Vitae

Ingrid Philippens werd op 3 juni 1964 geboren te Heerlen. Na haar middelbare school volgde zij een opleiding tot medisch analiste op de ZLS te Sittard en de OLAN te Nijmegen. Zij specialiseerde zich in de histo-/pathologie en behaalde in 1983 haar HBO-diploma. Een jaar later vervulde zij een functie als analiste op de Landbouwhogeschool (nu Landbouw-universiteit) te Wageningen in het kader van alternatieve dierproeven. Vervolgens behaalde zij het VWO diploma aan het Craneveldt College te Nijmegen, waarna zij in dienst trad als zoologisch analiste bij TNO-MBL in de afdeling farmacologie onder leiding van dr. O.L. Wolthuis. Na een aantal opleidingen in de sales, marketing en management begon zij in 1990 met de studie biologie aan de Rijksuniversiteit Utrecht. Zij specialiseerde zich in de neuro biologie. Haar stage, betreffende profylaxe tegen organofosfaatvergiftiging, volgde zij bij TNO en werd begeleid vanuit Utrecht door prof. dr. B.M. Spruijt van de vakgroep Medische Farmacologie en prof. dr. J.A.R.A.M. van Hooff van de vakgroep Ethologie. In 1994 behaalde zij haar doctoraalexamen Biologie. Geleidelijk ontwikkelde haar loopbaan binnen TNO zich van analiste naar wetenschappelijk medewerkster van de onderzoeksgroep neuro-farmacologie (hoofd: dr P.L.B. Bruijnzeel), wat ondermeer resulteerde in projectleiderschap van een aantal projecten. Een van deze projecten, gefinancierd door het Ministerie van Defensie, heeft geleid tot dit proefschrift.

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Dankwoord

Het in dit proefschrift beschreven onderzoek is uitgevoerd aan het voormalig TNO-MBL (TNO Medisch Biologisch Laboratorium) en TNO-PML (TNO Prins Maurits Laboratorium) en werd gefinancierd door het Ministerie van Defensie.
Bij het volbrengen van dit proefschrift is een aantal mensen tot steun geweest, dat ik bij deze hartelijk wil bedanken. Allereerst bedank ik Dr. Otto Wolthuis, die mij toendertijd bij TNO in dienst nam en mij de ruimte gaf voor verdere ontwikkeling via de studie Biologie. Hij is tevens de initiator van dit proefschrift. Bedankt dat ik heb mogen profiteren van jouw grote kennis en ervaring op het gebied van gedragsfarmacologie. Jouw enthiousiasme voor het vak werkte erg stimulerend. Typerend voor jou is dat je je naast kennis laat leiden door intuïtie Ondanks dat wetenschap staat voor het systematisch geordende geheel van het weten en van de regels waarmee verdere kennis verkregen kan worden, blijkt het wetenschappelijk moeilijk te omschrijven begrip "gevoel" een grote invloed te hebben. Dit werd reeds opgemerkt door een van onze grote wetenschappers Albert Einstein: „Ik vind nog altijd dat een kosmisch religieus gevoel de sterkste en meest verheven drijfveer tot wetenschappelijk onderzoek is". Otto uitte dit gevoel tijdens mijn sollicitatiegesprek waarbij hij mij voorstelde aan mijn toekomstige collega Raymond Vanwersch met de legendarische woorden: „Met hem zul je nog heel veel te maken krijgen in de toekomst". Hieruit bleek de kracht van zijn intuïtie, want deze collega bleek een 24 uurs collega te worden. En zonder deze collega had dit proefschrift waarschijnlijk niet bestaan. Naast Raymond waren er nog meer collega's die een bijdrage hebben geleverd aan dit proefschrift door hun inzet en goede collegialiteit. Met name wil ik Bas Groen voor zijn inzet, Dr. Bert Melchers voor zijn enthousiaste begeleiding, Ton van der Laaken voor zijn praktische ondersteuning en Dr. Ruud Busker voor zijn inhoudelijke bijdrage van het biochemische gedeelte bedanken. Bas wil ik bij deze tevens verontschuldigen dat ik zijn warmte in de vorm van koffie wel eens te veel liet afkoelen. Toch liet jij je daardoor niet ontmoedigen, waarvoor ik veel waardering heb. Dit geldt niet alleen voor de koffie, maar ook voor het experimentele werk. Jij maakte je vaak ongerust of de planning, soms per minuut, wel uitvoerbaar was, maar zie hier het resultaat. Bert, dat jij mijn co-promotor bent is natuurlijk al een bedankje waard. Ik moest aan jouw directe manier van communiceren wennen. Jouw opmerkzaamheid en scherpzinnigheid zijn mij vaak tot nut geweest. Daarnaast heb ik veel van jou geleerd, zoals jij met tegenslagen omgaat en je steeds weer staande weet te houden; mijn bewondering hiervoor. Nu ik de term co-promotor heb laten vallen wil ik vervolgens mijn promotoren bedanken, Prof. dr. Berend Olivier en Prof. dr. R.A.A. Maes. Hoewel het contact niet intensief was is al het noodzakelijke besproken waardoor de organisatie van dit proefschrift op een plezierige manier verlopen is. De communicatie verliep soepel en informeel. Tijdens mijn ontmoetingen met Berend heb ik het gevoel gekregen alsof ik hem al jaren ken. Berend, ook nog bedankt voor die heerlijke pannekoek. Binnen deze categorie wil ik ook Dr. Piet Bruijnzeel bedanken. Al was je geen co-promotor, toch heb ik dat het laatste halfjaar zo ervaren. Bedankt voor de snelle, punctuele correcties van dit proefschrift en het bruikbare commentaar. Opmerkelijk hoe jij je boven een tekst kan verplaatsen ongeacht het onderwerp. Ik hoop hier wat van geleerd te hebben. Hiernaast zag ik mij geruggesteund door de deskundige commentaren van Dr. Jan Langenberg, waarvoor ik hem wil bedanken. Dan zijn er natuurlijk nog de paranimfen die ik

wil bedanken, Jelly Zijlstra en mijn 24 uurs collega Raymond Vanwersch, geselecteerd op raakvlakken binnen TNO en binnen mijn familie. Jelly, doordat jij de wetenschap en kennis vanuit een "hoger" plan beschouwt, wierp jij een andere visie op mijn proefschrift. Ik heb er altijd veel plezier in beleeft om met jou hierover te praten. De onbaatzuchtige gedrevenheid die jij hierin uitte maakte op mij een grote indruk. Deze onbaatzuchtigheid blijkt tevens uit het feit dat jij met mijn broer Max de lakens deelt. Ik ben blij dat jullie gelukkig zijn en dat ik daarbij een bijdrage heb kunnen leveren. Natuurlijk bedank ik ook andere familieleden, mijn ouders en broers, die indirect medeverantwoordelijk zijn voor wie ik geworden ben. Verder wil ik de mensen bedanken die binnen het instituut op vele fronten voor onontbeerlijke ondersteuning zorgden, zoals de mensen van het secretariaat, archief, biotechniek, bibliotheek, automatisering, en presentatiedienst. Tot slot wil ik mijn kinderen bedanken, die een stimulans zijn voor alles wat ik doe.

11 december 1998, een grote dag in mijn leven. Niet alleen de dag zelf maar ook waar hij voor staat: het proefschrift een afronding, het huwelijk een begin.

Ingrid