Effectiveness of Physostigmine as a Pretreatment Drug for Protection of Rats from Organophosphate Poisoning

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Effectiveness of Physostigmine as a Pretreatment Drug for Protection of Rats from Organophosphate Poisoning. DESHPANDE, S. S., VIANA, G. B., KAUFFMAN, F. C., RICKETT, D. L., AND ALBUQUERQUE, E. X. (1986). Fundam. Appl. Toxicol. 6, 566-577. The effectiveness of physostigmine and atropine pretreatment against the lethal effects of sarin was studied in rats given lethal subcutaneous injections (130 μg/kg) of the organophosphate. Pretreatment of these animals with physostigmine 30 min prior to injection of sarin reduced mortality to 28% and when the drug coadministered with atropine only 4% of the animals died. The latter treatment also reduced significantly the extent and duration of symptoms due to sarin; however, atropine, pyridostigmine, and neostigmine injected alone did not protect animals against the lethal effects of sarin. Physostigmine caused only slight inhibition of cholinesterase in blood and skeletal muscle. Cholinesterase activity in blood and muscle of rats pretreated with physostigmine before sarin administration was significantly higher than in tissues from rats injected with sarin alone. In rats receiving sarin following pretreatment with physostigmine, twitch potentiation of extensor muscles and maintenance of tension during tetanic stimulation of the nerve recovered to near control levels. Muscle function recovered despite significant inhibition of cholinesterase. Effective protection against lethality by physostigmine could be related to protection of cerebral cholinesterase since inhibition of this enzyme by sarin was lowered significantly after pretreatment with physostigmine. Alternatively, physostigmine may also interact with the nicotinic acetylcholine receptor ion-channel complex directly. © 1986 Society of Toxicology.

Treatment of organophosphorus (OP) toxicity with the antimuscarinic agent, atropine, and certain oximes has been largely unsuccessful (Loomis and Salafsky, 1963; Heilbronn and Tolagen, 1965); however, pretreatment with reversible cholinesterase (ChE) inhibitors and atropine offers significant protection against diisopropylfluorophosphate (DFP; Koster, 1946) and soman (Berry and Davies, 1970; Gordon et al., 1978). The rationale for such protection is carbamylation of ChE to render it temporarily insensitive to irreversible inhibition by organophosphates. Although OP agents will phosphorylate irreversibly the remaining enzyme, decarbamylation of the protected enzyme results in adequate ChE activities in tissues for survival of animals.

The quaternary carbamates pyridostigmine, neostigmine, and the tertiary physostigmine have been studied for protection against the lethality of OP agents. Pyridostigmine and neostigmine have very limited access to the central nervous system due to the blood--brain barrier (Birtley et al., 1966) whereas physostigmine readily penetrates into the brain.

Results concerning protection against OP agent toxicity by carbamates have been conflicting because of species variation and different dose schedules. For example, physostigmine or neostigmine together with atropine raised the LD50 of soman eightfold in guinea pigs but offered only minimal protection to rats and mice (Berry and Davies, 1970). Pyridostigmine and physostigmine protect guinea
pigs (Gordon et al., 1978) and monkeys (Dirnhuber et al., 1979) to a similar extent against poisoning by soman. Apparently, protection of ChE peripheral blood and skeletal muscle with pyridostigmine itself is not adequate for protection of rodents against soman poisoning (Adler et al., 1984). Although neostigmine or physostigmine, in combination with atropine, were also unable to protect rats against lethal doses of soman (Harris et al., 1980), addition of the ganglionic blocking agent, mecamylamine, to the pretreatment regimen offered complete protection against soman lethality (Harris et al., 1980). This action of mecamylamine can be explained by its interaction with the acetylcholine receptor (AChR) and its associated channel as a competitive inhibitor (Ascher et al., 1979) and as a noncompetitive inhibitor of the AChR at neuromuscular synapses and certain areas of brain (Varanda et al., 1985; Schwartz et al., 1982). Although the mechanism for the protection against soman by the above agents remains unknown, results obtained with physostigmine indicate that central nicotinic and muscarinic ACh receptors may also be involved in OP agent poisoning.

The purpose of the present study was to compare three carbamates, physostigmine, neostigmine, and pyridostigmine, alone and in combination with low doses of atropine (sufficient to block secretions) for their ability to protect rats against a single lethal dose of the organophosphate agent sarin. In addition, ChE in whole blood, skeletal muscle, and brain was measured and compared with protection against the lethality of sarin and alterations in skeletal muscle function before and after pretreatment with physostigmine.

MATERIALS AND METHODS

Young female Wistar rats (200–220 g, 3 months old) were pretreated with physostigmine sulfate (100 μg/kg), neostigmine bromide (100 and 200 μg/kg), or pyridostigmine bromide (400 and 800 μg/kg) with or without coadministration of atropine sulfate (500 μg/kg) 30 min before injection of sarin (isopropylmethylphosphofluoridate, 130 μg/kg). All pretreatment drugs and sarin injections were administered subcutaneously. Although the data concerning lethality reported here were from a 24-hr observation period, it should be noted that the animals injected with 130 μg/kg sarin (a 100% lethal dose) died within 15 min of injection.

In vivo muscle contraction. Animals were anesthetized with chloral hydrate (400 mg/kg, ip). The distal tendon of the extensor muscle was released at the point of its insertion to the bone, and the sciatic nerve was cut about 2 cm above the popliteal region where it separates into the common peroneal nerve (supplying the extensor muscles) and the tibial nerve (innervating the triceps surae muscle). The two branches were separated carefully and ankle joints were clamped in a stereotaxic frame.

During the course of experiments, animals were kept warm with a lamp and the sciatic nerve was placed on a pair of bipolar silver electrodes. The distal tendon was attached to a force displacement transducer (Grass, FT.03) by means of a metal chain. Isometric twitch and tetanic contractions were recorded from muscles under a resting tension of about 5 g as described by Tiedt et al. (1978). Supramaximal pulses of 0.1 msec duration (delivered from a Grass stimulator S 88) stimulated the nerve kept moist by mineral oil. Muscle contractions were induced via continuous stimulation of the nerve at 0.1 Hz except during the trials of repetitive stimulation at 20 Hz for 10 sec and at 50 and 100 Hz for 2 sec. All responses were recorded on a Grass (7D) paper recorder. At the end of experiment the muscles were removed, blotted dry, and weighed on a torsion balance.

In vitro muscle contraction. The extensor muscles with nerve were removed from the rats anesthetized with chloral hydrate and perfused in a tissue bath. Desired concentrations of sarin were added to the perfusion medium. Isometric twitch and tetanic contractions were recorded in the same fashion as described for in vivo recording.

Biochemical analyses. A modification of the procedure by Ellman et al. (1961) was used to determine ChE activity in blood and tissues. Immediately prior to assay, blood was collected and diluted 1:10 in 0.1 M sodium phosphate buffer (pH 8.0). Blood, muscles, and brain were removed from animals anesthetized with ether and homogenized in the same buffer at 4°C using a polytron apparatus (Brinkmann Instruments) to give final concentrations of approximately 70 and 30 mg wet wt/ml, for muscle and brain, respectively. Homogenates (20 μl) were incubated for 1 hr at 37°C in 100 μl reagent containing 5 mM acetylthiocholine iodide. The reactions were stopped by adding 100 μl 6% perchloric acid; the protein precipitate was sedimented by centrifugation at 8000g for 2 min in a microfuge (Brinkmann, Model 3200). One hundred microliters of the supernatant was then added to 900 μl of a 1:15 dilution of Ellman's reagent and the optical density was determined at 412 nm. Unless otherwise specified, enzyme assays were performed in the presence of 10 μM.
Iso-ompa. All assays were linearly proportional with time for at least 60 min and with tissue ranging up to 1.0 mg muscle and 0.3 mg brain. Blanks contained all ingredients except tissue samples. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

_Solutions and drugs._ The physiological solution had the following composition (mmol/liter): NaCl, 135.0; KCl, 5.0; MgCl₂, 1.0; CaCl₂, 2.0; NaHCO₃, 15.0; Na₂HPO₄, 1.0; glucose, 11.0. This solution when bubbled with 95% O₂ + 5% CO₂ gas mixture had a pH of 7.2. Sarin and pyridostigmine bromide were obtained from the U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland. The stock solution (1 mg/ml) distributed in 100 or 200-μg quantities was kept frozen in sealed vials at −70°C. Dilutions were made fresh immediately before injection into the animals. All procedures involving the handling of sarin were carried out in a hood specially equipped with a charcoal filter system. Atropine sulfate, 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylthiocholine iodide, physostigmine sulfate, neostigmine bromide, tetraisopropyl pyrophosphoramide (Isoompa) were purchased from Sigma Chemical Company St. Louis, Missouri. All other reagents were of analytical grade.

Statistical analyses. Values are expressed as x ± SE. Statistical analysis for the difference in the mean values of the control and experimental data points was done using Student's t test.

**RESULTS**

_Carbamate Pretreatment and Protection from Lethal Effects of Sarin_

Administration of sarin (130 μg/kg) to adult female rats killed all of the animals within 15 min (Table 1). Pretreatment with atropine (500 μg/kg) alone reduced salivation and mucous secretions but did not prevent the lethal actions of sarin; however, pretreatment with physostigmine (100 μg/kg) alone protected rats against sarin-induced lethality (Table 1). Pretreated animals were protected not only up to 24 hr but also showed no further mortality over 10 days of observation. When atropine was used in combination with physostigmine, only 4% of the animals died. This beneficial effect of atropine was also observed in animals

<table>
<thead>
<tr>
<th>Pretreatment*</th>
<th>Dose (μg/kg)</th>
<th>No. animals dead/no. animals injected</th>
<th>% Lethality</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>—</td>
<td>66/66</td>
<td>100</td>
</tr>
<tr>
<td>Atropine</td>
<td>500</td>
<td>18/18</td>
<td>100</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>100</td>
<td>10/36</td>
<td>28</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>100</td>
<td>1/25</td>
<td>4</td>
</tr>
<tr>
<td>+ atropine</td>
<td>500</td>
<td>11/12</td>
<td>92</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>100</td>
<td>10/12</td>
<td>83</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>100</td>
<td>10/12</td>
<td>83</td>
</tr>
<tr>
<td>+ atropine</td>
<td>500</td>
<td>10/12</td>
<td>83</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>200</td>
<td>7/8</td>
<td>88</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>200</td>
<td>10/12</td>
<td>83</td>
</tr>
<tr>
<td>+ atropine</td>
<td>500</td>
<td>16/19</td>
<td>84</td>
</tr>
<tr>
<td>Pyridostigmine</td>
<td>400</td>
<td>11/15</td>
<td>73</td>
</tr>
<tr>
<td>Pyridostigmine</td>
<td>400</td>
<td>11/15</td>
<td>73</td>
</tr>
<tr>
<td>+ atropine</td>
<td>500</td>
<td>11/12</td>
<td>92</td>
</tr>
<tr>
<td>Pyridostigmine</td>
<td>800</td>
<td>13/18</td>
<td>72</td>
</tr>
<tr>
<td>Pyridostigmine</td>
<td>800</td>
<td>13/18</td>
<td>72</td>
</tr>
</tbody>
</table>

* Drugs used for pretreatment were injected subcutaneously 30 min prior to subcutaneous injection of 130 μg/kg sarin.

* These rats received 130 μg/kg sarin subcutaneously. All animals were observed for 24 hr for lethality.
pretreated with pyridostigmine where roughly 25% of the injected animals survived after sarin injection. Neostigmine given alone or in combination with atropine showed no protective effect. Attempts to increase neostigmine above 200 μg/kg were unsuccessful because this killed most animals. Doses of physostigmine less than 100 μg/kg provided no protection.

Overt responses of rats pretreated with physostigmine and atropine and subsequently challenged with sarin were significantly different from those rats injected with sarin alone. Very mild muscle fasciculations (spontaneous twitching movements) were the only symptoms observed during the 30 min after injection of physostigmine. Within 5 min after injection of a low dose of pyridostigmine (400 μg/kg) fasciculations were noted. A higher dose (800 μg/kg) produced fasciculations and tremor. Neostigmine produced similar effects as pyridostigmine. Convulsions were not observed with any of the carbamates. Rats injected with sarin alone showed typical symptoms of cholinergic crisis, including salivation, muscle fasciculations, severe tremors, convulsions, and gasping just before death. In contrast, all rats pretreated with physostigmine and atropine, and injected 30 min later with sarin, demonstrated no secretions and only moderate tremors during the first hour after sarin administration. Convulsions were observed in about 20% of these animals. Atropine and physostigmine-pretreated rats had no detectable weight loss or abnormal locomotion over a period ranging up to 10 days. Pyridostigmine was effective in prolonging survival of rats beyond 30 min; however, in contrast to physostigmine, these animals showed tremors and intermittent convulsions for 4–5 hr after sarin injection. Animals surviving at 24 hr after treatment with pyridostigmine showed 14% loss in body weight and demonstrated toe walking and sluggish righting reflex.

**Blood ChE**

Data in Table 2 show that sarin treatment alone reduced blood ChE activity by 90%. ChE

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Total ChE (μmol/ml/min)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (9)</td>
<td>0.89 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Physostigmine (9)</td>
<td>0.82 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Physostigmine + sarin (4)</td>
<td>0.26 ± 0.05</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>None (4)</td>
<td>0.94 ± 0.08</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Atropine (4)</td>
<td>0.99 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Atropine + sarin (4)</td>
<td>0.08 ± 0.04</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>None (9)</td>
<td>0.97 ± 0.07</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Physostigmine + atropine (9)</td>
<td>0.84 ± 0.03</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Physostigmine + atropine + sarin (4)</td>
<td>0.28 ± 0.04</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>None (4)</td>
<td>0.87 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Sarin (4)</td>
<td>0.10 ± 0.04</td>
<td>89</td>
</tr>
</tbody>
</table>

*Note.* Values are x ± SE of the number of experiments indicated in parentheses. Animals were injected subcutaneously with physostigmine (100 μg/kg) and atropine (500 μg/kg) alone and in combination 30 min prior to the subcutaneous injection of sarin. Blood was withdrawn from the tail vein before, 30 min after pretreatment, and 30 min after sarin injection except in groups 2 and 4 where blood was collected just before the animal died (~10 min).
in blood samples taken from rats 30 min after injection of physostigmine, either alone or in combination with atropine, was inhibited less than 15%; however, significant inhibition (~70%) occurred when physostigmine-pretreated animals were injected with sarin. Although this inhibition was large, it was less than that observed in animals not pretreated with physostigmine. The time course of recovery of blood ChE in rats protected by physostigmine pretreatment is shown in Fig. 1. Blood samples tested 1.5 hr after injection of sarin (i.e., 2 hr after pretreatment) showed a small but significant recovery in ChE. The enzyme activity returned progressively toward control values over the next 4 days. The time course of recovery of blood ChE was essentially identical in rats receiving physostigmine with or without atropine.

**Effect of Physostigmine Pretreatment on AChE Inhibition in the Extensor and Soleus Muscles**

In this series of experiments, AChE activities in extensor and soleus muscles were measured in rats pretreated with physostigmine and subsequently given a lethal dose of sarin. AChE activity in soleus muscles of the control group was approximately 35% lower than in extensor muscles (Fig. 2). AChE activity of muscles, removed prior to death (~15 min), from animals injected with sarin alone was only about 30% of that of control muscles (0.34 ± 0.07 vs 1.11 ± 0.01 nmol/mg in the extensor muscle and 0.21 ± 0.01 vs 0.72 ± 0.02 nmol/mg in the soleus muscle). Rats pretreated with physostigmine and given sarin had approximately 70% of AChE activity in both types of muscles at this time. Interestingly, muscles removed 30 min after injection of physostigmine (100 μg/kg) showed virtually no inhibition of AChE. The time course of recovery of tissue AChE over 5 days is shown in Fig. 3. Muscles examined at 2 hr and 1 day showed significant inhibition of AChE; however, in contrast to the pattern noted in blood, the enzyme did not recover completely in muscle (about 25% inhibition of AChE on Day 5).

**Blood ChE and Extensor Muscle and Brain AChE in Rats Receiving Sarin Following Pretreatment with Pyridostigmine or Physostigmine**

A comparison of effects of pyridostigmine and physostigmine pretreatment on the blood ChE and the muscle and brain AChE levels is shown in Table 3. It is obvious that pyridostigmine pretreatment (400 or 800 μg/kg) was unable to prevent extensive inhibition of AChE in the brain by sarin. In contrast, physostigmine pretreatment maintained brain AChE levels at a significantly higher level.

**In Vivo Recordings of the Effect of a Single Lethal Dose of Sarin on the Contractions of the Extensor Muscle**

A three- to four-fold potentiation of extensor muscle twitch and a significant reduction in the ability of the muscle to maintain tetanic
EFFECTIVENESS OF PHYSOSTIGMINE IN SARIN POISONING

FIG. 2. Effect physostigmine (PHY) and atropine (ATR) pretreatment on the extensor (open bars) and soleus (solid bars) muscles tissue AChE of rats injected with a lethal dose of sarin. Rats from the control (CON) group received a saline injection. Muscles from rats receiving sarin alone were removed just before death (~15 min) of the animals. Muscles from the remaining groups were removed 30 min (PHY and PHY + ATR) and 60 min (PHY + SARIN, PHY + ATR + SARIN) after pretreatment.

tension in vivo during 10 sec nerve stimulation at 20 Hz and for 2 sec at 50 Hz were observed 5 min after injection of 130 μg/kg sarin in an anesthetized rat. These effects are demonstrated in Fig. 4. The onset of potentiation of single twitches was observed at 5 min after injection of the OP agent and inability to sustain tetany developed within 7 min. The effects of pretreating rats with physostigmine and atropine 30 min before injection of sarin are shown in Fig. 5. Normal muscles maintained tetanic tension throughout the stimulation period of 10 or 2 sec for 20 or 50 Hz, respectively. There was approximately 40% potentiation of single twitches and a small reduction in the ability of the muscle to sustain tension at 20 Hz after administration of physostigmine and atropine (Fig. 5A). This effect was more pronounced 30 min after the injection of sarin. Such action was even more noticeable with tension obtained at 0.1 Hz (Fig. 5B) where there was a threefold potentiation of a single twitch. In addition to potentiation of single twitch responses, twitches elicited immediately after tetanus showed a marked depression. However, the post-tetanic depression of the muscle (i.e., depression of single twitch) recovered much faster than the tetanic response itself (Fig. 5C). Ninety minutes after injection of sarin, complete recovery from twitch potentiation and tetanic depression was seen (Fig. 5D). The mean contractile response in three experiments using physostigmine and atropine pretreatment is shown in Fig. 6. A marked increase in twitch tension and twitch/tetanus ratio and reduction in the final/initial tension ratio at 20 and 50 Hz occurred 30 min after injection of sarin in pretreated rats. All rats showed recovery in these parameters of muscle function over the next 1 hr.

Twitch potentiation and decline in muscle tension upon repetitive nerve stimulation after sarin also took place in vitro after adding sarin to the bath. Addition of sarin (200 nM) prevented muscles from sustaining tetanic contractions when stimulated at 50 and 100 Hz (Fig. 7). Although twitch responses were lower than those obtained under control conditions, complete recovery of tetanic tension and post-tetanic response was observed 60 min after washing the muscle with physiological saline.

DISCUSSION

Protection of Rats from Lethal Effects of Sarin

The results described in Table 1 demonstrate that physostigmine is effective in pro-

FIG. 3. Time course for inhibition of muscle AChE in the extensor (solid circles) and soleus (open circles) muscles of rats injected with physostigmine and atropine 30 min prior to the injection of sarin. 11 for broken line joining open or filled circles denotes injection of sarin. 11 for solid line indicates injection of physostigmine + atropine. 12 indicates injection of sarin.
TABLE 3
COMPARISON OF PYRIDOSTIGMINE AND PHYSOSTIGMINE PRETREATMENT AND SUBSEQUENT INJECTION OF A
LETHAL DOSE OF SARIN (130 µg/kg) ON BLOOD ChE AND MUSCLE AND BRAIN AChE LEVELS

<table>
<thead>
<tr>
<th>Pretreatment* (drug + dose)</th>
<th>Blood ChE (µmol/ml/min)</th>
<th>Extensor muscle AChE (nmol/mg wet wt/min)</th>
<th>Brain AChE (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.13 ± 0.01</td>
<td>0.20 ± 0.02</td>
<td>1.93 ± 0.46</td>
</tr>
<tr>
<td></td>
<td>(87)</td>
<td>(78)</td>
<td>(97)</td>
</tr>
<tr>
<td>Pyridostigmine (400 µg/kg)</td>
<td>0.38 ± 0.02</td>
<td>0.38 ± 0.05</td>
<td>2.65 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>(60)</td>
<td>(58)</td>
<td>(96)</td>
</tr>
<tr>
<td>Pyridostigmine (800 µg/kg)</td>
<td>0.36 ± 0.001</td>
<td>0.36 ± 0.02</td>
<td>3.21 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>(64)</td>
<td>(61)</td>
<td>(95)</td>
</tr>
<tr>
<td>Physostigmine (100 µg/kg)</td>
<td>0.28 ± 0.04</td>
<td>0.77 ± 0.05</td>
<td>28.7 ± 2.34</td>
</tr>
<tr>
<td></td>
<td>(71)</td>
<td>(31)</td>
<td>(56)</td>
</tr>
</tbody>
</table>

* Pretreatment with respective carbamates also included coadministration of 500 µg/kg atropine sulfate.

‡ Values are x ± SE of results obtained from three muscles in three rats. The numbers in parentheses are % of enzyme inhibition with respect to control. The enzyme activities for blood, muscle, and brain tissue in control saline-treated rats were 0.97 ± 0.07 µmol/ml/min, 1.07 ± 0.08 nmol/mg wet wt/min, and 65.9 ± 4.05 nmol/mg protein/min, respectively.

Protecting animals from the lethality of sarin. When used in combination with atropine, this drug offered almost complete protection in contrast to pyridostigmine, which showed significantly less protection than physostigmine.

Neostigmine was virtually ineffective. Previous studies with pyridostigmine (Dirnhuber and Green, 1978; Dirnhuber et al., 1979; Gordon et al., 1978) in guinea pigs, rhesus monkeys, and marmosets have shown that treatment with this carbamate raised the soman LD50 by a factor of 28 in monkeys, 15 in marmosets, and 8 in guinea pigs. Rats were least sensitive to the protective effect of pyridostigmine. Atropine given 15 sec after the administration of the organophosphate was essential for protection of all the species given pyridostigmine. In the absence of this supporting treatment, pyridostigmine was ineffective in protecting animals from the lethal effects of soman. In recent work with rats, pyridostigmine administered through Alzet osmotic pumps also offered no significant protection against soman poisoning (Adler et al., 1984). Data presented here show that physostigmine at a dose of 100 µg/kg provided significant protection against sarin poisoning in rats. This was greatly facil-

FIG. 4. Effect of subcutaneous injection of a lethal dose of sarin on the extensor muscle contractions recorded in vivo in a rat anesthetized with chloral hydrate (400 mg/kg IP). In this experiment twitch and tetanic responses of the muscle under control conditions were obtained in an anesthetized rat. Sarin was then injected subcutaneously. The rat died 15 min after injection of sarin. The nerve was stimulated at 0.1 Hz continuously except during repetitive stimulation at 20 and 50 Hz (see Methods).
The Role of Blood ChE and Muscle AChE Inhibition

Physostigmine alone (100 μg/kg) caused only 10% inhibition of blood ChE and virtually no inhibition of AChE in the extensor or soleus muscle. In contrast, injection of sarin in rats receiving no pretreatment with carbamates inhibited blood ChE by 90% and muscle AChE by 70%. Pretreatment with physostigmine (100 μg/kg), either alone or in combination with atropine, attenuated but did not prevent in-
FIG. 7. Effect of sarin on in vitro twitch and tetanic tensions in the extensor muscle. After obtaining control twitch and tetanic responses, the muscle was exposed to 200 nM sarin (shown by arrow). Note the inability of the muscle to sustain tetanic contractions at 50 and 100 Hz. After removal of sarin by repeated washing of the muscle, the ability of the muscle to sustain tetanus returns is shown in the last panel (the muscle was maintained at 30°C bath temperature). The nerve stimulation at 0.1, 20, and 50 Hz was as described in Fig. 4. At 100 Hz the nerve was stimulated for 2 sec.

Inhibition of blood ChE (Table 2). Similar protective or masking effects were also observed with AChE in extensor and soleus muscles (Figs. 2 and 3). Although the carbamate-type inhibitors protect the enzyme from irreversible phosphorylation by OP agents (Berry and Davies, 1970; Koelle, 1946; Fleisher and Harris, 1965), this mechanism alone probably cannot explain the protection of rats from the lethal effects of sarin. It is evident from the data shown in Fig. 3 that muscle AChE activity was significantly lower than control even at Day 5. In spite of the lower enzyme activity, the motor ability in these rats was hardly affected.

Previous studies (Harris et al. 1978, 1980; Harris and Stitcher, 1984) indicated that tertiary carbamates such as physostigmine were capable of penetrating the blood–brain barrier more readily than a quaternary carbamate such as pyridostigmine. Our finding that physostigmine was more effective than pyridostigmine in protecting rats from the lethal effects of sarin is in accord with this idea. Similarly, DFP-induced increases in bound and free ACh in brain tissue were reduced significantly by prior administration of physostigmine but not with pyridostigmine (Harris and Stitcher, 1984). Thus, protection against lethal doses of sarin by physostigmine could result from reversible inhibition of AChE. Reversal of this inhibition after sarin was inactivated could prevent excessive lethal accumulation of ACh. Results indicating that the extent of AChE inhibition in brain was reduced in three rats pretreated with physostigmine before administration of a lethal dose of sarin (Table 4) are in accord with this possibility.

Muscle Function in Rats Protected from Lethal Effects of Sarin

Soleus muscles from rats pretreated with physostigmine and injected with sarin were examined at 24 hr for possible electrophysiologic alterations at the endplate region (unpublished observations). These muscles
showed no membrane depolarization and values for the amplitude and frequency of MEPPs recorded at the endplate region were not significantly different from those obtained in control muscles. The only significant change was a roughly twofold prolongation in the decay phase of MEPPs with no alteration in the rise time. This prolongation of the decay phase could result from sarin-induced irreversible inhibition of a portion of AChE. Since the half-life of most of the nerve agents, including sarin, is very short, it is unlikely that the prolongation of decay phase of MEPPs observed at 24 hr could be a direct effect of sarin.

The contractions of the extensor muscle recorded in vivo showed three responses to the injection of sarin: potentiation of twitch response, failure to maintain muscle tension during repetitive nerve stimulation at 20 Hz, and an increase in twitch/tetanus ratio (Fig. 4). All of these effects returned to control levels within 2 hr in rats pretreated with physostigmine and atropine (Fig. 5 and 6). The possibility of a direct effect of the inhibitors on the neuromuscular junction is implied from the finding that removal of sarin from a muscle in vitro by repeated washing resulted in twitch potentiation, returning to control levels even though AChE remained inhibited 90%. These muscles were able to maintain tension during repetitive nerve stimulation at 20 and 50 Hz. Previous observations with pyridostigmine (Adler et al., 1984) and those made in the present study argue that other mechanisms in addition to AChE inhibition may also contribute to depression of tetanic tension after administration of sarin. Twitch potentiation by ChE inhibitors has been ascribed either to prolongation of the action of ACh at the postjunctional membrane, to generation of antidromic firing through prejunctional action of ACh, or through a direct action of these agents on the nerve terminals to initiate antidromic firing resulting in generation of multiple muscle action potentials (Clark and Hobbinger, 1983). AChE inhibition and resultant ACh accumulation is presumed to be the cause of tetanic fade. Prolonged EPPs, in the presence of anticholinesterase compounds tend to summate and cause depolarization of the postsynaptic membrane (see review by Hobbiger, 1976) and failure of neuromuscular transmission. Such failure could involve presynaptic actions as in the case of other agents such as VX (Rao and Albuquerque, unpublished results). If this hypothesis is correct, it is difficult to reconcile recovery in tetanic tension in the in vitro experiments after removal of sarin from the bath by washing (Fig. 7). Since 90% of the enzyme still remains phosphorylated at this time, spontaneous recovery of AChE does not appear probable.

Sufficient evidence has accumulated recently from voltage- and patch-clamp experiments showing weak agonist action of pyridostigmine, physostigmine, and soman (Akaike et al., 1984; Shaw et al., 1984) on ACh-activated receptor–channel complex. Physostigmine has also been shown to reduce mean channel lifetime (Shaw et al., 1984). Furthermore, all of these agents, by increasing the affinity of the agonist to its binding site, promote the formation of desensitized species (Sherby et al., 1985; Akaike et al., 1984; Shaw et al., 1983, 1984). Thus, it is likely that the cause of tetanic fade as observed in the present experiments could be the result of multiple direct effects of sarin on the endplate.

A number of noncholinergic effects of organophosphates have been observed and should be taken into account. Anticholinesterase agents such as sarin, DFP, and tabun have recently been demonstrated by Idriss and Albuquerque (1985) to interact with presynaptic nerve terminals of the glutamatergic synapses. Further, biochemical studies have demonstrated a paraoxon-dependent decrease in GABA levels in brain (Kar and Martin, 1972). Further, Lundy and Magor (1978) have shown that soman-induced seizures correlate with an increase in cGMP which has a function in GABA-ergic transmission. In a recent paper (Coudray-Lucas et al., 1984) physostigmine caused a decrease in striatal GABA while
the organophosphates paraoxon and soman did not.

In conclusion, only physostigmine offered significant protection of the rat from sarin lethality. This protection could be related to the ability of this tertiary agent to penetrate the central nervous system in contrast to the little penetration afforded by the quarternary compounds pyridostigmine and neostigmine. Further, physostigmine may have an additional molecular target which involves the ionic channel of the nicotinic receptor.

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REFERENCES


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