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The reversal by pyridostigmine of neuromuscular block produced by soman

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The effect of pyridostigmine on neuromuscular block produced by soman was studied in the isolated phrenic nerve-diaphragm preparation. In the rat, soman produced an irreversible reduction in tetanic tension and functional acetylcholinesterase (AChE) activity. Pretreatment with pyridostigmine before exposure of the diaphragm to soman, followed by removal of the anticholinesterase from the organ bath, produced a return of tetanic tension and an increase of 5% in functional AChE activity. Similar results were obtained in the guinea-pig. The changes in synaptic AChE activity were verified pharmacologically by showing a decrease in the blocking activity of acetylcholine in preparations pretreated with pyridostigmine in comparison to those given soman alone following removal of the anticholinesterase. The blocking dose of carbachol did not change in these two groups indicating that desensitization was not a component of the protective action. A comparison was also made of the results obtained by measuring inhibition of AChE in situ with those obtained from muscle homogenates. The implications of these results are discussed.

The successful use of the carbamate pyridostigmine as a pretreatment for soman (1,2,2-trimethyl propyl methylphosphonofluoridate) poisoning (Gordon et al 1978) has given rise to the need for a more complete understanding of the mechanism of its protective action. Berry & Davies (1970) suggested that the beneficial action of carbamates would depend on their property of being able to reversibly inhibit acetylcholinesterase (AChE EC 3.1.1.7) and upon the fact that tissues contain more AChE than is necessary for normal functioning. Carbamates have also been shown to produce a reduction in the number of post-synaptic acetylcholine (ACh) receptors and the amount of ACh released upon nerve stimulation when given by chronic administration (Chang et al 1973). A property that would be beneficial in the treatment of nerve agent poisoning.

Pyridostigmine, a quaternary carbamate which does not pass the blood-brain barrier (Birtley et al 1966) probably exerts its main protective action at a peripheral site, such as the neuromuscular junction. This has been studied by Dirnhuber & Green (1978) who showed that pretreatment with pyridostigmine was effective in reversing the neuromuscular block produced by soman, in a variety of animal species.

In the present study isolated phrenic nerve-diaphragm preparations of the rat and guinea-pig were used to elucidate whether the protective action of pyridostigmine at the neuromuscular junction was related to either the recovery of AChE from carbamoylation or to desensitization of the cholinoceptors, or to a component of both.

AChE activity was determined by a radiochemical method which measures the surface-located enzyme in the intact muscle in vitro. Previous work has shown that this method measures primarily the functional synaptic enzyme located at the end plate (Mittag et al 1971). The results were compared with those obtained on muscle homogenates using a conventional spectrophotometric method to give information on the total enzyme activity.

MATERIALS AND METHODS

Isolated phrenic nerve diaphragm preparation

The preparation was set up in a similar manner to that described by Bülbring (1946). The left hemidiaphragm of male Porton strain rats (250–300 g) and guinea-pigs (290–350 g) was mounted on an electrode holder and incubated in 100 ml Krebs buffer containing sodium acetate (1·0 g litre^{-1}) at 32 °C. The phrenic nerve was continuously stimulated with supramaximal square wave pulses (0·25 ms) at a frequency of 0·1 Hz with a Grass S6 stimulator. Every 15 min the preparation was tetanised at a frequency of 60 Hz for 5 s. Contractions were recorded from a baseline tension of 2–5 g, with a Devices force transducer type 4151 and a Devices MX2 recorder.

The effect of drug treatments (listed in Table 1) on the tetanic tension was monitored and related to the level of surface (functional) AChE and total AChE in

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Table 1. Summary of the experimental procedure used to relate changes in neuromuscular function with "functional" AChE in the phrenic nerve diaphragm preparation of the rat and guinea-pig.

Experiment and time (min)	Drug treatment	Samples for AChE assay: Time (min)	Volume (ml)
Control			
0	[1-^{14}C] ACh (5×10^{-6} M)	0, 30, 60, 75, 90	2
90	Wash out		
91	[1-^{14}C] ACh (5×10^{-6} M)	91, 105, 120, 150	2
Soman only			
0	[1-^{14}C] ACh (5×10^{-6} M)	0, 30, 60	2
60	Soman (40 or 100 nM)	75, 90	5
90	Wash out		
91	[1-^{14}C] ACh (5×10^{-6} M)	91, 105, 120, 150	5
Pyridostigmine* and soman			
0	[1-^{14}C] ACh (5×10^{-6} M)	0, 30	2
30	Pyridostigmine (1×10^{-7}–1×10^{-9} M)	60	2
60	Soman (40 or 100 nM)	75, 90	5
90	Wash out		
91	[1-^{14}C] ACh (5×10^{-6} M)	91, 105, 120, 150	5
Blank bath (No diaphragm)			
0	[1-^{14}C] ACh (5×10^{-6} M)	0, 30, 60, 90, 120, 150	5

* 1×10^{-4} M iso OMPA (tetraisopropylpyrophosphoramide) was added to the bath at 30 and 90 min in a limited number of experiments.

the same preparation using the methods described below.

The maintenance of a 60 Hz tetanic contraction for 5 s was the criterion used to assess normal neuromuscular function (Dirnhuber & Green 1978).

AChE assay

Surface AChE. The enzyme was measured radiometrically using the technique described by Mittag et al (1971). [1-^{14}C]Acetylcholine chloride, [1-^{14}C] ACh, 10·2–17·6 μ Ci μmole^{-1} (Amersham, Bucks, England) was added to the bath at a final concentration of 5×10^{-6} M and 2 or 5 ml aliquots removed at timed intervals (Table 1). The unhydrolysed [1-^{14}C]ACh was removed by extracting 3 times with an equal volume of the liquid cation exchanger Kalignost (3·75 g sodium tetraphenyl boron (BDH) in 250 ml ethyl butyl ketone (Pfaltz and Bauer Inc.)) as described by Fonnum (1969). The extraction efficiency was 99·5–99·9%. The [1-^{14}C]acetate remaining in the aqueous layer was made up to 5 ml with Krebs acetate ringer and added to 10 ml Insta-gel (Packard). The samples were counted in a Packard Tricarb model 3255. The counting efficiency was 80–83%.

The results were corrected for spontaneous hydrolysis of [1-^{14}C]ACh (3–5% of the control) and expressed as μmol acetate produced min^{-1} hemidiaphragm^{-1}. Acetyl-β-methylcholine, the specific substrate for AChE, was not used because its rate of hydrolysis was too low to accurately determine low levels of enzyme activity. Therefore the term AChE strictly refers to acetylcholine hydrolase activity of the tissue.

Total AChE

At the end of the experiment the hemi-diaphragm was homogenized in 0·1 M pH 8·0 phosphate buffer (1 : 5 w/v) and stored at −20 °C until assayed by the method of Ellman et al (1961). The results are expressed as μmol acetylthiocholine hydrolysed min^{-1} g^{-1} protein. Protein was determined by the method of Lowry et al (1951).

Effect of acetylcholine (ACh) and carbachol (CCh) on single twitches in the rat diaphragm

The % block of single twitches produced after 5 or 10 min by a standard dose of 5×10^{-3}, 1×10^{-4} M ACh or 1×10^{-4} M CCh was determined in diaphragms treated with soman only and in diaphragms which had pyridostigmine pretreatment followed by soman, using the dose schedules described in Table 1.

The results of all experiments are expressed as the mean with s.d. for 4 or 6 experiments.

RESULTS

Relationship between protection of tetanic tension depressed by soman obtained with pyridostigmine pretreatment and AChE activity

a. *Surface AChE*

Rat. The lowest dose of pyridostigmine required to completely reverse tetanic tension depressed to >95% by 40 nM soman was 1×10^{-7} M (Fig. 1). Lower doses (1×10^{-9}–5×10^{-8} M) produced an incomplete recovery. There was no reversal in diaphragms given soman alone.

The changes in tetanic tension were compared with the levels of surface AChE, measured by the hydrolysis of [1-^{14}C]ACh in the same preparation. In control diaphragms the hydrolysis of [1-^{14}C]ACh was linear over the time used in the experiment. The rate of hydrolysis was 1·16 ± 0·05 n mol min^{-1} before the wash out and 0·98 ± 0·07 n mol min^{-1} after the wash out. In all subsequent experiments each diaphragm was used as its own control by measuring the hydrolysis of [1-^{14}C]ACh during the first 30 min of the experiment.

The effect of pyridostigmine pretreatment followed by soman in comparison to soman alone on the level of surface AChE is shown in Fig. 2. The addition of 1×10^{-7} M pyridostigmine to the bath reduced the surface AChE by 22 ± 2·4% but had no effect on single twitch or tetanic tension. The addition of 40 nM soman reduced the enzyme activity to <5% of the control activity. When the preparation was

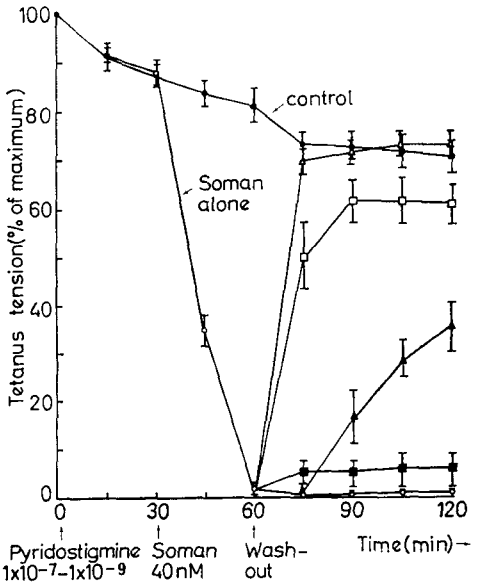

FIG. 1. The effect of different concentrations, 1×10^{-7} (△), 5×10^{-8} (□), 1×10^{-8} (▲), 1×10^{-9} M (■), of pyridostigmine on the recovery of tetanic tension depressed by soman in the rat. Each point represents the mean with s.d. of 4 experiments.

washed to remove the excess soman and pyridostigmine the AChE activity returned to 24 ± 2·8% of the control value over 60 min. Return of tetanic tension occurred gradually over 15 min after wash out and was associated with a level of 5% surface AChE activity above the soman control. Diaphragms treated with soman alone had 9·9 ± 1·2% surface AChE activity after the wash out which remained constant over 60 min. There was a highly significant difference ($P < 0.001$) in the levels of surface AChE between these two groups after wash out.

The 9·9% surface AChE present after wash out in diaphragms given soman alone could be reduced to 2·1 ± 0·9% (n = 5) either by the addition of a second dose of 40 nM soman or by the addition of 1×10^{-4} M iso-OMPA, a selective inhibitor of cholinesterase (ChE EC 3.1.1.8) (Heffron 1972).

Guinea-pig. Similar results were obtained with the guinea-pig diaphragm. 100 nM soman completely depressed tetanic tension in 30 min. The neuromuscular block could be reversed to control levels by pretreatment with 1×10^{-7} M pyridostigmine. Return of tetanic tension occurred gradually over 15 min after wash out and was associated with a level of 8% surface AChE activity above the soman control.

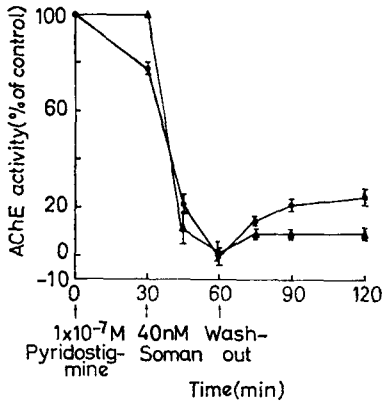

FIG. 2. The effect of pyridostigmine pretreatment followed by soman in comparison to soman alone on the level of surface AChE activity in the rat diaphragm. Each point represents the mean ± s.e.m. of 6 experiments. Pyridostigmine + soman (●), soman alone (▲).

b. *Total AChE*

A comparison of the total AChE activity in the diaphragm after soman treatment and pyridostigmine pretreatment followed by soman in the rat and guinea-pig is summarized in Table 2. There was a significant difference between the two groups in the rat ($P < 0.001$) but not in the guinea-pig ($P > 0.05$).

Effect of acetylcholine and carbachol on single twitches

The results are summarized in Table 3. In a control diaphragm 5×10^{-3} M ACh produced no effect on single twitch after 5 min whereas in diaphragms treated with 40 nM soman 1×10^{-4} M ACh when added 30 min after removal of excess soman produced a complete block of single twitch after 2 min. This effect was reversed by washing. In comparison, 1×10^{-4} M ACh produced only a 26% block of single twitch in diaphragms pretreated with pyridostigmine before soman.

Table 2. Summary of the % inhibition of total AChE 60 min after removal of the anticholinesterase agents in diaphragms treated with soman in comparison to those treated with pyridostigmine followed by soman in the rat and guinea-pig. The results are means with s.d. of 6 experiments.

Animal	Control value μ mol min^{-1} g^{-1} protein	% Inhibition of total AChE* soman only	Pyridostigmine + soman
Rat	5·97 (0·77)	94·7 (2·82)	75·8 (6·48)†
Guinea-pig	4·90 (0·80)	84·3 (6·80)	91·8 (3·20)

* The term AChE refers to total acetylthiocholine hydrolase activity.

† Significantly different from soman only $P < 0.001$.

Table 3. Effect of acetylcholine and carbachol on single twitches in the rat diaphragm. The results are the means with s.d. of 4 experiments.

Experiment	Time (min)	Tetanic tension	Effect of ACh Dose	Effect of ACh % tension 5 min after addition of ACh dose	Effect of CCh Dose	Effect of CCh % tension 10 min after addition of CCh dose
Control	0′	Normal	—	—	1×10^{-4}M	5·3 (4·5)
	30′ after wash out	Normal	—	—	1×10^{-4}M	22·1 (8·0)
Soman only*	0′	Normal	5×10^{-5}M	100	1×10^{-4}M	1·3 (1·3)
	30′ after wash out	Depressed by >95%	1×10^{-4}M	0 at 2 min	1×10^{-4}M	13·6 (7·2)
Pyridostigmine* + soman	0′	Normal	5×10^{-5}M	100	1×10^{-4}M	6·5 (5·4)
	30′ after wash out	Normal	1×10^{-4}M	73·7 ± 16·2	1×10^{-4}M	22·6 (6·1)

* Dosing schedules as described in Table 1.

Carbachol produced a complete block of single twitch in control preparations using a dose of 1×10^{-4} M. The same dose produced a slightly reduced response 90 min later indicating that some desensitisation had occurred. The addition of soman or pyridostigmine followed by soman produced no effect on the standard carbachol response produced 30 min after removal of the excess anticholinesterase agents.

DISCUSSION

The results of the present investigation provide experimental evidence that pyridostigmine pretreatment protects the neuromuscular junction from irreversible block by soman, by carbamoylating a portion of the AChE. After removal of excess soman spontaneous decarbamoylation produces sufficient free AChE to restore neuromuscular function.

Attempts to relate protection of the animal to levels of AChE have been hampered in the past by the methods of enzyme assay which require removal and homogenization of the tissue before assay of AChE activity. During this procedure the carbamoylated enzyme may undergo spontaneous hydrolysis and produce a falsely high value. This problem was overcome in the present study by using a radiochemical method to measure the surface or 'functional' AChE in the isolated diaphragm preparation. This assay, originally developed by Mittag et al (1971) is a non-destructive method in which pharmacological responses can be correlated with the effect on the enzyme in the same preparation.

Using this technique it was shown that pretreatment with pyridostigmine would reverse tetanic tension depressed to >95% by soman in the rat. The return of tetanic tension to the control level occurred 15 min after removal of the excess soman by washing and this was associated with a recovery of surface AChE from <5% to 14·8 ± 1·5% of the control value. This value increased to 24 ± 2·8% over sixty minutes. In comparison treatment with soman alone produced a complete tetanic block and this was associated with 9·9 ± 1·2% surface AChE 15 min after washing. This level remained constant for 60 min but could be reduced to 2·1 ± 0·9% either by the addition of a second dose of soman or by incubation with iso-OMPA. This indicates that most of the background enzyme activity was probably ChE since it is known that the rat diaphragm contains both AChE and ChE (Davison 1953) which will hydrolyse ACh and that ChE does not play any part in neuromucsular transmission (Heffron 1972).

These results support previous observations on muscle homogenates (Barstad 1960) and indirect calculations (Barnes & Duff, 1953; Meeter & Wolthuis 1968; Hobbiger 1976) that low levels (between 1–10%) of AChE are necessary for the maintenance of normal neuromuscular function.

The differences in synaptic AChE reported were verified pharmacologically by demonstrating that a standard dose of ACh was less effective in producing a neuromuscular block in diaphragms pretreated with pyridostigmine, in comparison with those given soman alone. It was also shown that protection of neuromuscular function by pyridostigmine was not due to desensitization of the cholinoceptors because the blocking dose of carbachol did not change. The detection of changes in synaptic AChE activity by comparing the blocking activity of ACh and CCh has previously been reported by Danilov et al (1976).

Similar results were obtained using guinea-pig diaphgrams. This suggests that the mechanism of action of pyridostigmine reversal of neuromuscular blockade produced by soman was similar in the two species. However, it is known that there is a marked species difference in response to the protection afforded by carbamate pretreatment; guinea-pigs respond better than rabbits and rats are virtually unresponsive (Gordon et al 1978). Therefore this result would support the suggestion that the lethal action of soman in the rat is due predominantly to a

central action (Meeter & Wolthuis 1968; Dirnhuber & Green 1978).

The results obtained by measuring surface AChE activity were compared with measurements of total acetylthiocholine hydrolase activity in the same diaphragms. There was a significant difference between the enzyme activity in the 2 groups in the rat but not in the guinea-pig. This may be due to the fact that a higher dose of soman was used in the guinea-pig and that the contribution of internal AChE to the total hydrolysis of ACh is different in the two species. This clearly demonstrates the advantage of using a non-destructive method of measuring surface AChE in comparison to tissue homogenates. One possible limitation of this method is that the rate of hydrolysis of [^{14}C]ACh may be limited by diffusion of the substrate into the extracellular space and synaptic clefts (Mittag et al 1971), and therefore this technique must be used only in the comparison of relative enzyme activities (Hobbiger 1976).

In conclusion it has been shown that pyridostigmine pretreatment was effective in reversing the neuromuscular block produced by soman in the isolated phrenic nerve diaphragm preparation of the rat and guinea-pig. This protection was related to a recovery of 5–8% of the surface AChE activity above the soman control.

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