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THE EFFECT OF ACETYLCHOLINESTERASE-INHIBITION ON THE TONUS OF GUINEA-PIG BRONCHIAL SMOOTH MUSCLE

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Abstract—The irreversible acetylcholinesterase inhibitor soman (O-[1,2,2-trimethylpropyl]-methylphosphonofluoridate) induced contraction of guinea-pig primary bronchial smooth muscle. The apparent affinity (ED_{50}) of acetylcholine (ACh) was altered from control value of 12 μM to 0.3 μM following exposure of the bronchial smooth muscle to 14 μM soman for 15 min *in vitro*. The ED_{50} of the cholinergic agonist carbachol was not changed even when the acetylcholinesterase (AChE) activity was inhibited completely. The intrinsic activity (α) of ACh and carbachol was not significantly changed after exposure to soman for 15 min. The results demonstrate that the effect of soman is only due to its anticholinesterase activity. Furthermore, the contraction induced by histamine was not altered by concentrations of soman which increase the cholinergic stimulation. This indicates that histamine does not induce contraction of bronchial smooth muscle in guinea pig through the release of ACh or by modulation of muscarinic receptors. Furthermore, soman also inhibited the carboxylesterase activity in the primary bronchi. In respiratory tissue this group of enzymes may have a major protective function, due to their ability to bind several organophosphorus compounds. Compared to studies performed on other species, this study shows that guinea-pig bronchi are very sensitive to the AChE-inhibitor soman. Therefore, exposure to very low concentrations of AChE-inhibitors may induce contraction of bronchial smooth muscle.

The acute toxic effects of organophosphates are primarily mediated by an irreversible inhibition of acetylcholinesterase (AChE), resulting in local accumulation of acetylcholine (ACh). The extensive use of organophosphates increases the risk of human exposure. The important route of exposure is by inhalation and following exposure the cause of death is anoxia due to a combination of factors; severe bronchoconstriction, excess accumulation of bronchial and salivary gland secretions, weakness or paralysis of the accessory muscles of respiration and sudden respiratory paralysis.

Previous studies have shown that the organophosphorus compound soman (O-[1,2,2-trimethylpropyl]-methylphosphonofluoridate) has a substantial effect on the rat bronchial smooth muscle [1, 2]. Inhibition of AChE-activity by soman exposure *in vitro* or by inhalation exposure *in vivo* increases the synaptic concentration of ACh and thereby the stimulation of muscarinic receptors with subsequent contraction of bronchial smooth muscle. Similar results have been found in tracheal smooth muscle isolated from adult mongrel dogs, where soman potentiated the cholinergic stimulation by inhibition of AChE-activity. This potentiation was not due to stimulation of muscarinic receptors [3]. There is an obvious variability in the innervation of the airways and lung among species of animals, and extrapolation from one species to another, with regard to either physiologic responses or anatomic distribution of the nerves should be made with caution [4]. The small and large airways from guinea-pig, dog and man respond with contraction on exposure to histamine [5]. Airway smooth muscle from the rat, on the other hand, is not sensitive to histamine, but is contracted by 5-hydroxythryptamine (5-HT) [6]. This is in agreement with the results showing that the anaphylactoid reaction in rat is primarily mediated by 5-HT and not by histamine [7]. Previously, in rat and dog the classical mast-cell degranulating agent 48/40 has shown to produce a severe anaphylactoid reaction, but only a negligible effect is seen in guinea pig and man [8].

To elaborate on the effect of soman on the airway smooth muscle we have used the primary bronchi from the guinea pig. The guinea-pig airways, unlike rat and dog airway smooth muscle, have been shown to have many similarities with the human airways where histamine in addition to ACh play an important role as a mediator of contraction [5].

Previously soman has been shown to inhibit AChE-, ChE- and carboxylesterase (CarbE) activities in respiratory tissue as well as in other peripheral tissues in rat following exposure to soman by inhalation [2, 9]. In respiratory tissue CarbE may have a major protective function, due to their ability to bind several organophosphorus compounds [10, 11]. This may be of great importance since the lung is the primary route of entry for these compounds.

The aim of the present work was to study the effect of soman on the cholinergic nervous system in guinea-pig bronchial smooth muscle. Secondly, it was of interest to investigate whether soman altered the contraction induced by histamine. A third objec-

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tive was to determine the importance of the CarbE enzymes in the protection of AChE in bronchial smooth muscle.

MATERIALS AND METHODS

Animals

Male guinea pigs (MOL:DH) within the weight range 200–300 g were used. The animals were given a standard laboratory diet and given water *ad lib*. The guinea pigs were without symptoms of infections in the respiratory system. They were examined at the National Institute of Public Health, Oslo, Norway.

Chemicals

Acetylcholine chloride, atropine sulphate and histamine dihydrochloride were purchased from Norsk Medisinal Depot, Oslo, Norway. Carbachol and tetrodotoxin was from Sigma Chemical Co. (Poole, U.K.).

Soman (O-[1,2,2-trimethylpropyl]-methyl-phosphonofluoridate, assessed to be more than 99% pure by nuclear magnetic resonance spectroscopy, was synthesized in this laboratory.

Physiological methods

Following decapitation and dissection the left and right primary bronchi were mounted in parallel and contraction was measured as a reduction of the diameter of the airway [6]. The thermostatically controlled organ-bath contained Krebs buffer (50 ml, 37°) of the following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.6; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; KH_2PO_4, 1.2; glucose, 11.1 (pH = 7.4). The solution was gassed with 95% O_2 + 5% CO_2. For electrical stimulation the bronchi were mounted between platinum electrodes and stimulated by a Grass S88 Stimulator. The preparations were given a preload of 1.6 g and equilibrated for 60 min before the start of the experiments. The preload of 1.6 g was selected because the contraction response to a maximal stimulation with carbachol was rapid and gave a high and constant level of contraction. Carbachol was used as the reference agonist ($\alpha = 1.0$) since carbachol was most potent. The contractions were recorded isometrically by Grass Force Displacement Transducers (FT 03C) and monitored on a Grass Polygraph (Model 7) fitted with amplifiers (7 P 1A).

Acetylcholine, carbachol and histamine were added by cumulative application, since there were no differences in the apparent affinities (ED_{50}) when the agonists were added by single or by cumulative application.

Soman was added to the *in vitro* preparation and left for 15 min before it was removed by washing with the Krebs buffer.

Biochemical methods

Determination of acetylcholinesterase (AChE) and pseudocholinesterase (ChE) activities. Following the physiological experiments (approximately 6 hr) the guinea-pig bronchi were frozen (−20°) and later homogenized (10% w/v) in 20 mM sodium–potassium phosphate buffer (pH = 7.4) (glass/glass homogenizer, 20 strokes, 720 rpm, ice cold) before the enzyme activity determinations. Total ChE-activities were determined by the radiochemical method of Sterri and Fonnum [12] at 30°. AChE-activity was measured after inhibition of ChE-activity with ethopropazine [13].

Determination of carboxylesterase (CarbE) activity. The activity of CarbE, which hydrolyze 4-nitrophenylbutyrate, was determined at 30° by a spectrophotometric method [14]. The assay mixture consisted of 0.1 M sodium phosphate buffer pH = 7.8, 2 mM 4-nitrophenylbutyrate and tissue homogenate in a total volume of 3.0 ml. The stock solution of 4-nitrophenylbutyrate was 0.6 M in acetone. Optical absorbance of 4-nitrophenol at 400 nm was followed in a Beckman DU-50 Spectrophotometer fitted with kinetics Soft-Pack module, with the assay mixture omitting the tissue homogenate as reference. A molar absorption coefficient of 17,000 $M^{-1}\,cm^{-1}$ was used [15]. Protein content was determined by the Method of Lowry [16].

Statistics

Data are given as mean ± SEM. Statistical analyses were performed with the Student's *t*-test (two-tailed).

Table 1. The effect of soman on acetylcholinesterase (AChE), pseudocholinesterase (ChE) and carboxylesterase (CarbE) activities in the guinea-pig primary bronchial smooth muscle

Soman (M)	Enzyme activities ($\mu mol \times min^{-1} \times mg\ protein^{-1} \pm SEM$) AChE	ChE	CarbE	N
0	$3.5 \pm 0.6 \times 10^{-3}$ (100 ± 17)	$2.8 \pm 0.5 \times 10^{-3}$ (100 ± 18)	$4.1 \pm 0.3 \times 10^{-2}$ (100 ± 7)	5
1.4×10^{-9}	$4.4 \pm 0.7 \times 10^{-3}$ (126 ± 20) ns	$2.2 \pm 0.2 \times 10^{-3}$ (76 ± 8) ns	$2.7 \pm 0.4 \times 10^{-2}$ (66 ± 10)*	8
1.4×10^{-8}	$5.4 \pm 1.3 \times 10^{-4}$ (16 ± 4)**	$3.0 \pm 0.4 \times 10^{-4}$ (11 ± 1)**	$2.4 \pm 0.3 \times 10^{-2}$ (57 ± 7)**	4
1.4×10^{-7}	$1.7 \pm 0.4 \times 10^{-4}$ (5 ± 1)**	$1.9 \pm 0.3 \times 10^{-4}$ (7 ± 1)**	$1.3 \pm 0.3 \times 10^{-2}$ (32 ± 7)**	6
1.4×10^{-6}	0	0	$3.6 \pm 0.1 \times 10^{-3}$ (9 ± 1)**	4
1.4×10^{-5}	0	0	0	5

Acetylcholinesterase (AChE), pseudocholinesterase (ChE) and carboxylesterase (CarbE) activities in guinea-pig primary bronchi after treatment with soman *in vitro*. The bronchi were exposed to soman for 15 min in a physiological buffer prior to recording of the contraction induced by cholinergic and histaminergic stimulation. Following the physiological experiments the enzyme activities were measured as described in Materials and Methods. The results are expressed as mean ± SEM and resulted in differences from controls at $^{**}P < 0.01$, $^{*}P < 0.05$, ns $P > 0.05$. The results in parenthesis are in per cent of control.

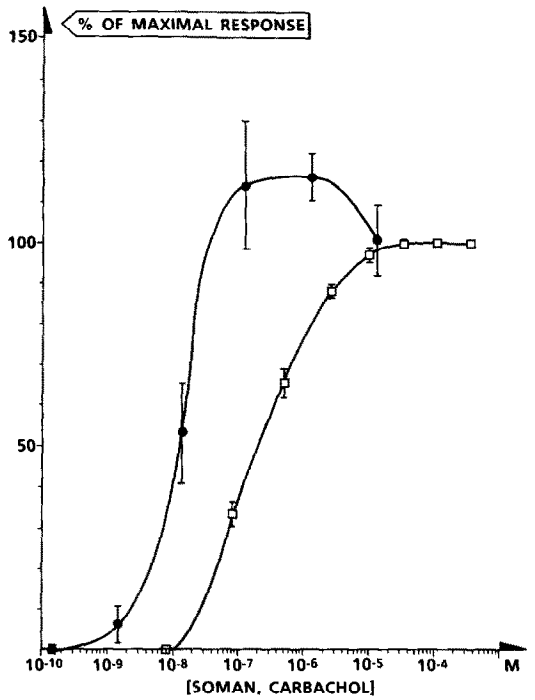

Fig. 1. Concentration–response curves for soman (●, N = 6) and carbachol (□, N = 37) on the isolated primary bronchi from guinea pig. The responses to soman and carbachol are mean ± SEM of N experiments and plotted in per cent of the maximal response to carbachol before exposure to soman.

RESULTS

The results show that guinea-pig primary bronchi were very sensitive to soman since concentrations of soman in the nanomolar range enhanced the contractions induced by ACh and electrical stimulation. Soman present alone in concentrations lower than 1 nM produced no contractions and maximal response was reached at approximately 100 nM of soman (Fig. 1). The response to application of soman was rapid and the contraction was irreversible in the absence of a muscarinic antagonist. The maximal contraction induced by soman was in the same range as was seen for carbachol (Fig. 1). The spontaneous increase in muscle tension induced by soman was antagonized by atropine and the concentration-dependent response to soman correlated well with the inhibition of AChE-activity (Table 1). Complete inhibition of AChE-activity occurred after *in vitro* exposure to a soman-concentration of 140 nM for 15 min.

The intrinsic activities of ACh and carbachol were in the same range, whereas the intrinsic activity of histamine was larger ($\alpha = 1.25 \pm 0.1$, N = 9) compared to carbachol ($\alpha = 1.0$). There was no alteration of the apparent affinity (ED_{50}) to carbachol (Table 2) or histamine (Table 2) following exposure to soman (1.4 nM–14 μM). In comparison, soman substantially reduced the ED_{50} of ACh from 12 μM to 0.3 μM (Table 2). A low concentration of soman (14 nM) enhanced the response to cholinergic stimulation of the guinea-pig bronchial smooth muscle shown by

Table 2. The effect of soman on apparent affinity (ED_{50}) and intrinsic activity (α) of acetylcholine, carbachol and histamine in guinea-pig bronchi

Soman (M)	ED_{50} (M)			α'			α''		
	Acetylcholine	Carbachol	Histamine	Acetylcholine	Carbachol	Histamine	Acetylcholine	Carbachol	Histamine
0	$1.2 \pm 0.6 \times 10^{-5}$(32)	$2.6 \pm 0.4 \times 10^{-7}$ (31)	$2.1 \pm 0.3 \times 10^{-6}$(30)	1.00(32)	1.00(31)	1.00(30)	1.00(32)	1.00(31)	1.00(30)
1.4×10^{-9}	$2.8 \pm 2.3 \times 10^{-5}$ns(4)	$1.3 \pm 0.8 \times 10^{-6}$ns(6)	$3.8 \pm 0.6 \times 10^{-6}$ns(5)	1.21 ± 0.02^{ns}(4)	1.14 ± 0.10^{ns}(6)	1.14 ± 0.12^{ns}(5)	1.25 ± 0.06^{ns}(4)	1.21 ± 0.09^{ns}(6)	1.20 ± 0.13^{ns}(5)
1.4×10^{-8}	$5.5 \pm 1.0 \times 10^{-7}$**(5)	$2.1 \pm 1.3 \times 10^{-7}$ns(6)	$1.8 \pm 0.4 \times 10^{-6}$ns(7)	1.41 ± 0.16^{ns}(5)	0.98 ± 0.17^{ns}(6)	0.86 ± 0.14^{ns}(7)	1.87 ± 0.27^{ns}(5)	1.51 ± 0.23^{ns}(6)	1.29 ± 0.12^{ns}(7)
1.4×10^{-7}	$2.0 \pm 0.5 \times 10^{-7}$**(7)	$1.5 \pm 0.5 \times 10^{-6}$*(7)	$4.2 \pm 0.4 \times 10^{-6}$ns(5)	0.71 ± 0.06^{ns}(7)	0.31 ± 0.03*(7)	0.29 ± 0.08**(5)	1.75 ± 0.13^{ns}(7)	1.42 ± 0.18^{ns}(7)	1.02 ± 0.13^{ns}(5)
1.4×10^{-6}	$2.9 \pm 1.2 \times 10^{-7}$**(8)	$1.6 \pm 0.4 \times 10^{-6}$*(6)	$2.7 \pm 0.8 \times 10^{-6}$ns(6)	0.70 ± 0.08^{ns}(8)	0.58 ± 0.11^{ns}(6)	0.30 ± 0.07**(6)	1.76 ± 0.13^{ns}(8)	1.75 ± 0.15^{ns}(6)	1.07 ± 0.11^{ns}(6)
1.4×10^{-5}	$3.0 \pm 1.4 \times 10^{-7}$**(6)	$1.1 \pm 0.5 \times 10^{-6}$ns(6)	$5.5 \pm 2.0 \times 10^{-6}$ns(5)	0.34 ± 0.06*(6)	0.16 ± 0.03**(6)	0.21 ± 0.08**(5)	1.46 ± 0.14^{ns}(6)	1.16 ± 0.10^{ns}(6)	1.04 ± 0.06^{ns}(5)

The intrinsic activities of acetylcholine, carbachol and histamine were recorded before and after exposure to soman. α' is the intrinsic activity of the respective agonist after exposure to soman, while α'' is the intrinsic activity of the agonist including the activity of soman. The α'- and α''-values are relative to control in the absence of soman, which is given an $\alpha = 1.00$. The results are expressed as mean ± SEM of the number of experiments shown in brackets and resulted in differences from control at **$P < 0.01$, *$P < 0.05$, $^{ns}P > 0.05$.

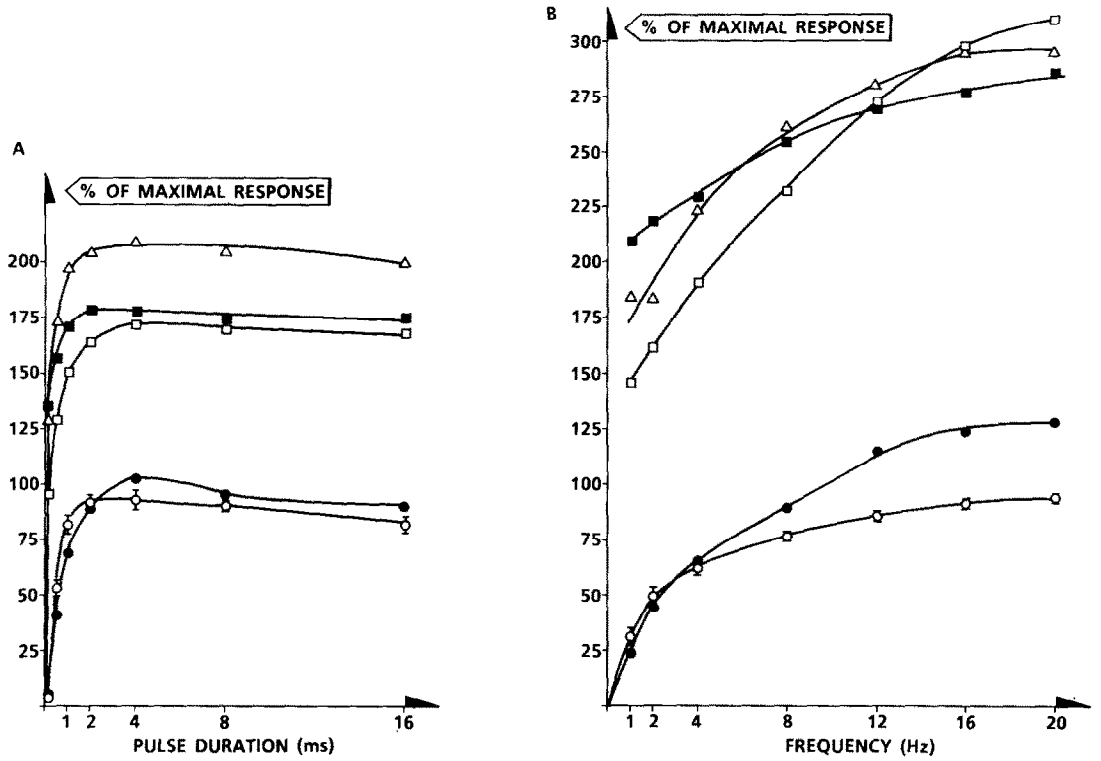

Fig. 2. Effect of electrical stimulation of the isolated guinea-pig primary bronchial smooth muscle. Part A shows the effect of varying the pulse duration (0.1–16 msec) and keeping the pulse frequency constant (10 Hz). Part B shows the effect of varying the pulse frequency (1–20 Hz) with a constant pulse duration (6 msec). The responses are mean ± SEM of N experiments and plotted in per cent of control stimulation without soman (○, N = 29); soman; 1.4 nM (●, N = 5), 14 nM (□, N = 3), 140 nM (■, N = 7), 1.4 μM (△, N = 6).

the decrease in ED_{50} of ACh from 28 to 0.55 μM.

Soman had no effect on the contraction induced by maximal stimulation with ACh, carbachol or histamine when the spontaneous increase in muscle tension induced by soman was included in the response (α'') (Table 2). However, exposure to soman reduced the maximal contraction (α') induced by the agonists when the intrinsic activity was not corrected for the soman-induced contraction (Table 2).

As shown in Table 1, in addition to inhibit AChE-activity soman also decreased the activities of pseudocholinesterases (ChE) and carboxylesterases (CarbE) in the guinea-pig bronchi (Table 1). There was a concentration-dependent inhibition of the ChE- and CarbE-activities, with an inhibition of 89 and 43% respectively following exposure to 14 nM of soman for 15 min. The most pronounced decrease in enzyme activities occurred following exposure to 14 nM of soman. A complete inhibition of AChE- and ChE-activities was seen following 15 min exposure to approximately 140 nM.

Low concentrations of soman (14 nM) also enhanced the efficacy of electrical field stimulation of the muscle (Fig. 2). It was previously shown that electrical stimulation release ACh and thereby stimulate to contraction [6]. It was blocked by atropine (1 μM) and tetrodotoxin (1 μM) (not shown). High concentrations of soman (micromolar range), however, induced contraction and therefore reduced the contraction response following electrical field stimulation. Together soman and electrical field stimulation enhanced the tension of guinea-pig bronchi severalfold (Fig. 2). Exposure to soman enhanced the contraction induced by increasing both the frequency and the duration of stimulation.

DISCUSSION

Soman is a specific irreversible AChE-inhibitor [17] and therefore a good model substance for studying the effect of AChE-inhibitors in the respiratory system. The present results show a concentration-dependent inhibition of AChE- and ChE-activities and a good correlation between the esterase-activities and the tonus of the guinea-pig bronchial smooth muscle. The results are in good agreement with previous results showing substantial effects of soman on the cholinergic nervous system in rat bronchial smooth muscle [1] and tracheal smooth muscle from mongrel dogs [3]. The inhibition of the AChE-activity was, however, more pronounced in the guinea-pig bronchial smooth muscle, indicating a higher sensitivity of AChE to soman in this species. A higher apparent affinity (ED_{50}) of ACh in guinea-pig bronchial smooth muscle compared to rat [1] was also seen, giving further evidence for the higher sensitivity of the cholinergic muscarinic receptors in

guinea-pig bronchial smooth muscle to cholinergic stimulation. It is therefore of importance to explore the effect of AChE-inhibitors in several species, before extrapolation of the sensitivity in man is drawn.

The results of this study have clearly shown that it is only the cholinergic nervous system which is altered by soman. Since the effect of soman was completely blocked by atropine, there was no evidence of stimulation of muscle cells *per se*. Furthermore, since the fact that soman did not change the ED_{50} or the maximal contraction induced by histamine, the result implies that neither soman nor ACh interact with histamine receptors. These results clearly show that although histamine is an important mediator of contraction in guinea-pig and human airways [5], histamine does not modulate the effect of ACh, at least not in the guinea-pig bronchial smooth muscle.

In the presence of soman (140 nM–14 μM) a decrease in the intrinsic activity (α') of ACh, carbachol and histamine was seen (Table 2). The results with ACh and carbachol are in agreement with results on the bronchi isolated from albino rats [1]. The reduced effects of ACh, carbachol and histamine in the presence of soman is probably due to inhibition of AChE-activity and the subsequent accumulation of spontaneously released ACh followed by stimulation of muscarinic receptors. The release of endogenously synthesised ACh is shown during electrical stimulation. The intrinsic activity (α''), which is the summation of the contraction induced by both the agonists and soman, was not different from control. This implies that soman does not enhance the maximal contraction of bronchial smooth muscle induced by ACh, carbachol and histamine, but only increases the sensitivity to cholinergic stimulation.

The data from electrical stimulation support the hypothesis that the soman-induced contraction was mediated by accumulation of ACh. Previously electrical stimulation has been shown to induce release of ACh from rat bronchial smooth muscle [6] and it has also been shown to be inhibited by tetrodotoxin and atropine. The most pronounced effect on electrical stimulation was seen following exposure to 14 nM soman. This correlates well with the inhibition of AChE-activity which was substantial at this concentration. A similar enhancement of electrical stimulation was seen in rat [1], although the effect during high frequency stimulation was much smaller. These results may also indicate that guinea-pig bronchi is more sensitive to AChE-inhibition than rat bronchi. As in the rat bronchial smooth muscle, at high concentrations (micromolar range) of soman, a sustained contraction of the guinea-pig bronchial smooth muscle was seen. This is due to the complete inhibition of AChE-activity and accumulation of spontaneously released ACh and a following stimulation of muscarinic receptors.

Previously carboxylesterases (CarbE), which is a group of several isoenzymes, has been shown to bind organophosphorus compounds irreversibly. It is therefore suggested that these enzymes can act as scavengers and reduce the ability of organophosphates to inhibit AChE [10, 11, 18, 19]. Following exposure of rats to soman *in vivo* [9, 20] or rat bronchial smooth muscle *in vitro* [1] both AChE- and CarbE-activities were inhibited to a large extent. CarbE were, however, less inhibited, which means that soman reached the target enzyme, AChE, although CarbE was present. This may indicate that CarbE play a less important role in respiratory tissue compared to other tissues in preventing soman from inhibiting the AChE-activity.

In conclusion, the results of this study show that guinea-pig bronchial smooth muscle AChE-activity is more sensitive to inhibition by soman than AChE in rat bronchi and mongrel dog tracheal smooth muscle. Furthermore, the results show that inhibition of AChE-activity by soman does not enhance the guinea-pig bronchial smooth muscle sensitivity to histamine. The CarbE enzymes does not prevent soman from inhibiting the AChE-activity in guinea-pig bronchi and they may therefore constitute a poor protection to soman in the respiratory organ.

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