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EFFECTS OF ORGANOPHOSPHATES ON PRESYNAPTIC EVENTS IN THE VASCULARLY PERFUSED PHRENIC NERVE-HEMIDIAPHRAGM PREPARATION FROM THE RAT

R. A. ANDERSEN,*† D. MALTHE-SØRENSSEN, E. ODDEN and F. FONNUM

Division for Environmental Toxicology, Norwegian Defence Research Establishment, P.O. Box 25, N-2007 Kjeller, Norway, and * Department of Toxicology, National Institute of Public Health, Geitmyrsveien 75, 0462 Oslo 4, Norway

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Abstract—A vascularly perfused phrenic nerve–hemidiaphragm preparation from the rat was developed to study effects of physostigmine and some organophosphate inhibitors on the synthesis and release of endogenous and deuterium-labelled (choline—D_9) acetylcholine (ACh) as well as the presynaptic uptake of choline. Choline and ACh were determined by combined gas chromatography/mass spectrometry. Without stimulation the endogenous levels of ACh were 320 pmole/hemidiaphragm for unlabelled and <1 pmole/hemidiaphragm of deuterium-labelled ACh. After stimulation at 15 Hz for 1 hr, 460 pmole/hemidiaphragm of unlabelled and 15 pmole/hemidiaphragm of deuterium-labelled ACh were found. Without stimulation the release of unlabelled ACh was 6 pmole/min/hemidiaphragm and for deuterium-labelled 0.2 pmole/min/hemidiaphragm. Evoked release (15 Hz, 1 hr) was 22 pmole/min/hemidiaphragm for unlabelled and 1.8 pmole/min/hemidiaphragm for deuterium labelled ACh. During stimulation and treatment with high concentrations (10^{-5}–10^{-4} M) of soman, DFP and Vx the level of unlabelled endogenous ACh increased, but the level of deuterium labelled ACh decreased in the diaphragm. During stimulation and treatment with these inhibitors the release of both unlabelled and labelled ACh decreased. During treatment with high concentrations (10^{-5}–10^{-4} M) of sarin and physostigmine there were no changes in endogenous levels or release of unlabelled or deuterium labelled ACh. The different effects of cholinesterase inhibitors are probably linked to the synthesis and release mechanism of ACh rather than to the choline uptake mechanism.

The isolated rat phrenic nerve–hemidiaphragm represents an almost ideal preparation for studies of a cholinergic synapse. This preparation has therefore been extensively used in studies of various aspects of synaptic transmission [1–8]. Today there is an increasing demand for reliable *in vitro* methods for testing of pharmaca and other substances especially in neuropharmacology and toxicology. Several modifications of the rat hemidiaphragm preparation have therefore been developed. In previous work by Bierkamper *et al.* [5, 6, 9, 10] and Endeman and Brunengraber [7], vascularly perfused hemidiaphragms were used, while Alkon *et al.* [11], Gundersen and Jenden [12, 13], Miledi *et al.* [14–16] used unperfused preparations. In contrast to previous work [5–7, 9, 10], this paper describes a vascular perfusion technique performed while the preparation was suspended in a temperature-regulated chamber containing moist oxygen plus 5% CO_2. During retrograde perfusion through the inferior phrenic vein, perfusate emerging from the surface of the preparation was collected as drops through a funnel beneath the suspended preparation. To ensure a proper flow resistance throughout the capillary bed a part of the body wall was left surrounding the diaphragm muscle.

Lipophilic cholinesterase inhibitors seem to cause formation of the ACh pool called surplus ACh both in ganglia and diaphragm [17]. It has previously been proposed that the highly lipophilic organophosphate soman has direct action on both pre- and postsynaptic membrane ACh receptors of neuromuscular junctions besides its inactivation of cholinesterase [18]. In a preliminary study we noted differences in the release of ACh by the two organophosphorous anticholinesterases sarin and soman. This has prompted us to investigate the effect of the carbamate physostigmine and of the organophosphate inhibitors soman, DFP, Vx and sarin, all different in lipophilic properties, on synthesis and release of ACh in the cholinergic synapse in the vascularly perfused hemidiaphragm preparation. Both unlabelled and deuterium labelled ACh were used to enable more detailed studies of the kinetics involved. Presynaptic uptake of choline was also looked into. Vascular perfusion of the synaptic area of the rat hemidiaphragm will probably cause a more efficient removal of accumulated ACh in the synaptic cleft than might be possible in bath experiments. ACh was measured as described previously [19, 20] by combined gas chromatography/mass spectrometry by the use of deuterated choline isotopes as internal standards.

MATERIALS AND METHODS

Chemicals. The cholinesterase (ChE) inhibitors diisopropoxyphosphorylfluoride (DFP), pinacolyl-

† Author to whom correspondence should be addressed.

oxymethyl phosphorylfluoride (soman), isopropoxymethyl phosphorylfluoride (sarin) and O-ethyl S-2-diisopropyl aminoethylmethylphosphonothiolate (Vx) were prepared and the purities controlled by nuclear magnetic resonance spectroscopy to be more than 98% (see Andersen *et al.* [21] for chemical formulas of ChE inhibitors as well as for bimolecular inhibition rate constants for ChE preparations obtained from various animal species). The carbamate physostigmine salicylate, cholinebromide and acetylcholine bromide were obtained from Sigma Chemical Co., while hemicholinium-3 (Hc-3) was bought from Aldrich. The choline isotopes AChCl(N-$(CD_3)_3$), (ACh-D_9), 98% purity, and ChCl(N-$(CD_3)_3$), (Ch-D_9), 98% purity were supplied by Kor isotopes, Cambridge, MA, while ACh-1,1,2,2-D_4Br, ACh-D_{13}Br and Ch-D_{13}Br were purchased from Merck, Sharp & Dohme, Montreal, Canada.

It was important that the chemicals used for ACh and Ch extraction as well as those used for processing for GC/MS analysis were of high grade purity. These chemicals are therefore listed together with their respective article production numbers: acetic acid (63), ammonium hydroxide (5428), citric acid (244), dichloromethane (6048) isopropylalcohol (993), methanol (6000 and 6009), methylethylketone (9708 as well as Baker 8052), molecular sieve (5705), N-pentane (7179), perchloric acid (517), potassium carbonate (4928), Reinecke-salt (7552), toluol-4-sulphonic acid (silver salt) (818000), thiophenol (808159) and toluene (8325) were all bought from Merck, acetonitrile (15.460-1) from Aldrich, dibasic ammonium citrate (27171) and potassium acetate (29581) from British Drug House, propionylchloride (81970) from Fluka and tetramethylammoniumbromide (T-7012) from Sigma Chemical Co.

Perfusion fluid. A Tyrode solution of the following composition was used to perfuse vascularly the isolated phrenic nerve–hemidiaphragm: NaCl 8.0 g/l (137 mM), $NaHCO_3$ 1.0 g/l (12 mM), KCl 0.2 g/l (2.7 mM), $CaCl_2$ 0.2 g/l (1.8 mM), $MgCl_2$ 0.1 g/l (1 mM), NaH_2PO_4 0.05 g/l (0.4 mM) and dextrose 20 g/l (11 mM).

The solution was saturated with a mixture of O_2 (95%) and CO_2 (5%). The Tyrode solution was kept at approximately 37° while perfused. Our choice of perfusion fluid is based on extensive studies of the phrenic nerve–hemidiaphragm preparation in our laboratory using organ baths containing Tyrode [2].

Animal housing and maintenance. The rats used in the present experiments were kept in macrolon cages and maintained in 12 hr light and 12 hr darkness at 20° and 55% humidity for at least 3 weeks

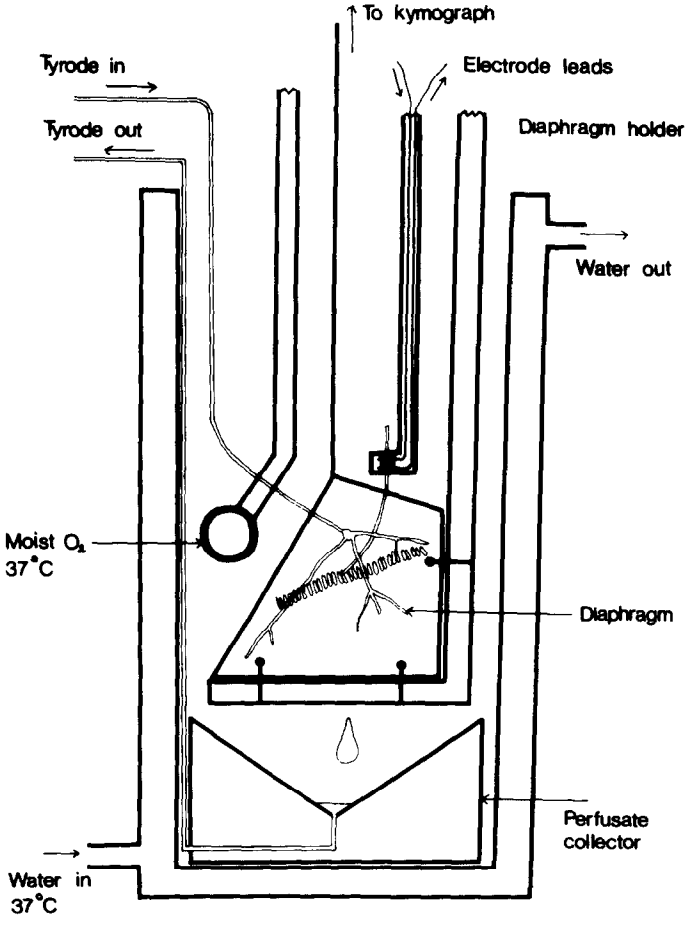

Fig. 1. The vascularly perfused phrenic nerve–hemidiaphragm preparation from the rat and experimental arrangement. See also description in the text.

prior to sacrifice. They were fed *ad libitum* with a commercial pelleted diet (Ewos, Södertälje, Swéden) and had free access to water.

The vascularly perfused phrenic nerve–hemidiaphragm preparation. Isolated phrenic nerve–hemidiaphragms (left side) from adult albino rats of both sexes (Wistar strain) weighing from 250 to 320 g, were suspended in a temperature regulated 150 ml organ chamber supplied with moist O_2 containing 5% CO_2 as illustrated in Fig. 1. The average wet weight of the hemidiaphragms used was 260 mg ± 50 (mean ± SD). The temperature in the organ chamber as well as in the gas were kept at 37 ± 0.5° throughout the experiments. The hemidiaphragm was stimulated indirectly and supramaximally through the phrenic nerve with rectangular electric pulses from an electronic pulse generator. The single pulse duration was 0.3 msec and the stimulation frequency used was generally 15 Hz. Muscle contractions were recorded on a kymograph apparatus supplied by Braun, Melsungen, F.R.G. The writing lever employed had approximately 10 to 1 ratio of advantage. The load on the hemidiaphragm was 6–8 g.

According to Greene [22] the vascular system of the diaphragm is rather complex (Fig. 2). The blood is supplied through the musculophrenic artery, the pericardiacophrenic artery and through the superior and inferior phrenic arteries. The venous system consists of the superior and inferior phrenic veins. The phrenic nerve–hemidiaphragm preparation was perfused retrogradely through the inferior phrenic vein. This vein collects blood from the inferior surface of the diaphragm [22]. Therefore, all parts of the hemidiaphragm might not be perfused equally well. In order to prevent the perfusion fluid shunting through blood vessels possessing low perfusion resistance, some of the chest wall together with a small part of the spine were left surrounding the diaphragm muscle. A thorough examination shortly (5 min) after initiation of the perfusion revealed no traces of red blood cells left in the hemidiaphragm. As shown in Fig. 1, perfusate (in 1 ml fractions), after emerging from the cut hemidiaphragm arteries, was collected as drops falling into a Perspex funnel beneath the suspended preparation inside the organ chamber. The perfusion fluid was supplied to the hemidiaphragm through a Formocath polyethylene tube 0.25 mm inner and 0.50 mm outer diameter bought from Becton, Dickinson & Co, Rutherford, U.S.A. Both supply as well as withdrawal of perfusion fluid from the funnel were performed by the use of a Gilson Minipuls 2 perfusion pump. Total volume of the perfusion circle was 0.3 ml. The perfusion rate was 0.2 ml/min, which is about twice the normal blood flow in musculature (11–58 ml/100 g/min according to Catchpole and Gersch [23]). The viability of the perfused preparation (Tyrode only) was found to be beyond 8 hr.

After being properly mounted in the preparation chamber, each hemidiaphragm was equilibrated with continuous perfusion with Tyrode only, for at least 30 min. Following equilibrium the inhibitor treatment was initiated. When cholinesterase was to be irreversibly inhibited by the organophosphorus compounds, these were included in the perfusion fluid in proper concentrations for 30 min, while the carbamate physostigmine, because of its reversible nature of inhibition, was kept present in the perfusion fluid throughout the whole experiment.

After 30 min of anticholinesterase treatment choline-D_9 in a concentration of 10^{-6} M was added continuously to the perfusion fluid throughout the experiment. Following 20 min of equilibration with the choline isotope six perfusate fractions were initially collected at resting conditions, then 12 frac-

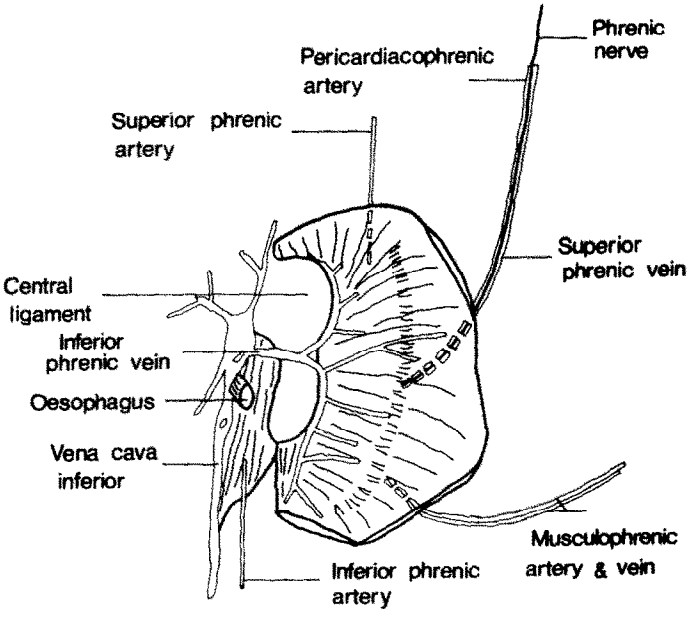

Fig. 2. A sketch of the anatomy of the hemidiaphragm showing the localization of the phrenic nerve, endplate zone and blood vessel system. The preparation was perfused retrogradely through the inferior phrenic vein.

tions (each 1 ml) were collected while the hemidiaphragm was stimulated through the phrenic nerve, for a total stimulation time of 1 hr. In some experiments without stimulation all 18 fractions were collected for reference.

We avoided keeping the organophosphorus inhibitors present during the whole experiment since it has been found [14] that continued presence of DFP in unperfused preparations produced undesirable effects. For instance, DFP reduced the amplitude of miniature endplate potentials through a postsynaptic action and also produced a presynaptic block in the propagation of impulses to some endplates. These effects were, however, reversible.

The anticholinesterases were prepared and kept in distilled water or in isopropyl alcohol on ice as 10^{-2} M stock solutions for no longer than 10 min. They were then properly diluted 10–100 times and immediately added to the perfusion fluid. Physostigmine was repeatedly replaced by fresh solution during the experiment.

Cholinesterase activity was measured directly in hemidiaphragm homogenates by the Ellman method [24] and by the radiochemical method of Fonnum [25].

Extraction of ACh and Ch. Immediately after collection of each fraction 2 ml of ice-cold 0.6 M $HClO_4$ were added. Then the mixture was shaken and stored frozen at −80°. At the end of each experiment the diaphragm preparation itself was frozen in liquid nitrogen. While frozen the tissue surrounding the hemidiaphragm including the phrenic nerve was removed. The remaining hemidiaphragm muscle was then immersed in 1 ml ice-cold 0.4 M $HClO_4$ and stored frozen (−80°). Before extraction of ACh the hemidiaphragm was thawed and homogenized by the use of a polytron blendor (Kinematica, Luzern, Switzerland). The homogenate was then centrifuged at 15,000 *g* for 20 min at 4°. To all collected fractions including the prepared hemidiaphragm supernatant 0.5 nmole ACh-D_4 was added as an internal standard.

Acetylcholine was extracted mainly as described by Zsilla *et al.* [19, 20] and by Jenden and Hanin [27]. To the supernatants obtained as described above were added 180 μl 7.5 M potassium acetate to adjust pH to 4.2–4.4. After centrifugation of the supernatants at 35,000 *g* for 20 min at 4° the choline esters were precipitated as Reinecke salts by adding 50 μl of 10 mM tetraethylammoniumchloride and 3 ml 2% ice-cold Reinecke salt to each supernatant. After 1 hr the mixture was centrifuged at 1000 *g* for 10 min at 4°. The precipitate was saved and freeze-dried.

For regeneration of ACh as tosylate the freeze-dried samples were mixed with 300–600 μl 5 mM silver tosylate/acetonitrile. After centrifugation at 1000 *g* for 2 min at 4° the supernatants to be obtained should be a clear solution, if not the procedure was repeated with more tosylate added. The supernatants were then transferred to screwcapped 1 ml microflex tubes.

To convert the ACh to its tertiary amine derivative for gas chromatography/mass spectrometry analysis 200 μl 50 mM Na–benzenethiolate in dry methylethylketone was added to each microflex tube. Traces of O_2 were carefully removed with N_2 gas before the tubes were capped. The tubes were then shaken, incubated at 80° for 45 min and cooled to room temperature.

Extraction and concentration of tertiary amines were performed by addition of 20 μl 0.5 M citric acid and 200 μl *n*-pentane to each sample. The tubes were then well shaken and centrifuged at 1000 *g* for 2 min. The pentane layer on top was removed, 200 μl pentane was added again and the procedure repeated. Traces of pentane were removed by blowing with N_2 gas. Then 75 μl dichloromethane and 20 μl base (113 *g* ammoniumcitrate, dibasic + 127 ml 7.5 M ammoniumhydroxyd to 250 ml aq. dest) were added to each tube. After mixing, the upper layers were transferred to new microflex tubes. The samples were then ready for injection.

Total recovery of the overall ACh assay was estimated to be about 75%, for the Reinecke salt precipitation in particular about 85%. The lower detection limit was found to be less than 1 pmole per sample.

The mass fragments, characterized by the *m/z* values 58, 60, 64 and 66, were continuously recorded in the multiple ion monitoring mode of a LKB 2091 mass spectrometer equipped with a Pye Unicam gas chromatograph. The gas chromatographic separation was performed on a 6 ft ¼ in. glass column packed with Pennwalt 223 + 4% KOH on chromosorb AW, 70–100 mesh.

Analysis conditions were: column temp 185°, injector temp 195°, separator temp 260°, ion source temp 280°, carrier gas flow (helium) 20 ml/min, retention time of the choline esters 112 sec, electron energy 22 eV.

Statistics. For data evaluation in the present work we used the Student's *t*-test. Values of *t* correspond to $P = 0.05$ (95% confidence interval). The numerical values as well as curve points given are based on mean values of at least 4 independent determinations.

RESULTS

For our experimental conditions the highest values of incorporation of the deuterium labelled choline (Ch-D_9), both in endogenous ACh and in ACh released, were found when the isotope was present in the perfusion fluid at the concentration of 10^{-6} M. Therefore this Ch-D_9 concentration was routinely used as standard in the experiments. Likewise stimulation of the phrenic nerve at 15 Hz gave the highest levels of ACh present endogenously in the diaphragm, the highest release of ACh and the highest incorporation of the choline isotope (Table 1). The tetanic contractions during higher stimulation frequencies might prevent access of perfusion fluid into the diaphragm.

Treatment for 30 min with the inhibitors used in the concentration of 10^{-6} M depressed more than 98% of the cholinesterase activity in the hemidiaphragm preparations. In accordance, the preparations showed pronounced high frequency inhibition (Wedensky inhibition) during a 5 sec period of electrical stimulation of the phrenic nerve at frequencies of 60 and 120 Hz. The preparations also showed pronounced increase in height of single con-

Table 1. Values of endogenous and released ACh for the rat hemidiaphragm at the end of the stimulation period (fraction 18) corresponding to 1 hr stimulation for different stimulation frequencies. Incorporated levels of Ch-D_9 are also included

Stimulation (Hz)	ACh-D_9 (pmole/ hemidiaphragm)	Endogenous ACh (pmole/ hemidiaphragm)	Incorporated (%)	Released ACh-D_9 (pmole/min/ hemidiaphragm)	Released ACh (pmole/min/ hemidiaphragm)	Incorporated (%)
0	<1	320 ± 20	<0.3	0.2 ± 0.1	6.4 ± 0.2	3.1
1	5 ± 2	330 ± 33	1.5	0.2 ± 0.1	6.3 ± 0.3	3.2
5	10 ± 1.5	530 ± 18	1.8	1.2 ± 0.2	16 ± 0.7	7.5
15	15 ± 2	460 ± 15	3.2	2.0 ± 0.2	21 ± 0.6	9.5
20	12 ± 2	430 ± 20	2.8	1.6 ± 0.3	18 ± 0.7	8.9

The experiments refer to the presence of 10^{-6} M Ch-D_9 in the perfusion fluid and to treatment of the hemidiaphragm with physostigmine at the concentration of 10^{-5} M. The values given are based on at least four independent determinations and are expressed as mean ± SD. For further details, see Methods.

Table 2. Values of endogenous ACh in the stimulated hemidiaphragm (15 Hz) after treatment with organophosphates

Inhibitor conc. (M)	Soman			DFP			Vx			Sarin			Soman + Hc-3 (10^{-5} M)		
	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%
10^{-4}	385 ± 29	3 ± 1*	0.6	384 ± 19*	4 ± 1*	1.0	449 ± 42*	3 ± 2*	0.7	322 ± 8	20 ± 2	6.2	—	—	—
10^{-5}	379 ± 22	13 ± 2*	3.4	305 ± 21	7 ± 1*	2.3	386 ± 31	11 ± 2	2.8	339 ± 18	18 ± 4	5.3	106 ± 17	2 ± 1.5	1.9
10^{-6}	291 ± 34	23 ± 3	7.9	284 ± 23	24 ± 3	8.5	285 ± 21	18 ± 3	6.3	291 ± 14	21 ± 4	7.2	74 ± 11	<2	<2.7

Values measured at the end of the stimulation period (fraction 18 corresponding to 1 hr stimulation). The experiments refer to the continuous presence of 10^{-6} M Ch-D_9 in the perfusion fluid (in combination with Hc-3 when used). D_9 and D_0 refer to ACh with and without deuterated choline incorporated, respectively. The results are given in pmole ACh/hemidiaphragm and are based on at least four independent determinations. Values are expressed as mean ± SD. Asterisks indicate significant differences compared to values obtained for 10^{-6} M inhibitor concentration ($P = 0.05$) determined by the Student's *t*-test. For further details, see Methods.

trations at low stimulation frequencies [26]. Inhibition not as high as 95% gave a reduction or absence of ACh release. Uninhibited hemidiaphragms were never found to give detectable ACh levels in the perfusates. During long term electrical stimulation of the phrenic nerve, in the inhibited condition, muscle contractions were greatly attenuated even though ACh was released and had reached a steady plateau. Muscle contractions returned, however, following 5 min of rest. These observations are in accordance with previous results by Bierkamper and Goldberg [5].

As control inhibitor of acetylcholinesterase we used 10^{-5} M physostigmine present in the perfusion fluid throughout the experiment. For this inhibitor without stimulation the endogenous levels of ACh were 320 pmole/hemidiaphragm for unlabelled and <1 pmole/hemidiaphragm for deuterium-labelled ACh. For stimulation with 15 Hz for 1 hr the levels were higher, namely 460 pmole/hemidiaphragm of unlabelled and 15 pmole/hemidiaphragm of labelled ACh (see Methods for experimental design). The percentage of labelled to unlabelled endogenous ACh was calculated to be <0.3 without stimulation and about 3 with stimulation (Table 1). The values were not found to vary appreciably for physostigmine in the concentration range 10^{-6}–10^{-4} M.

The effects of pretreatment of the hemidiaphragm

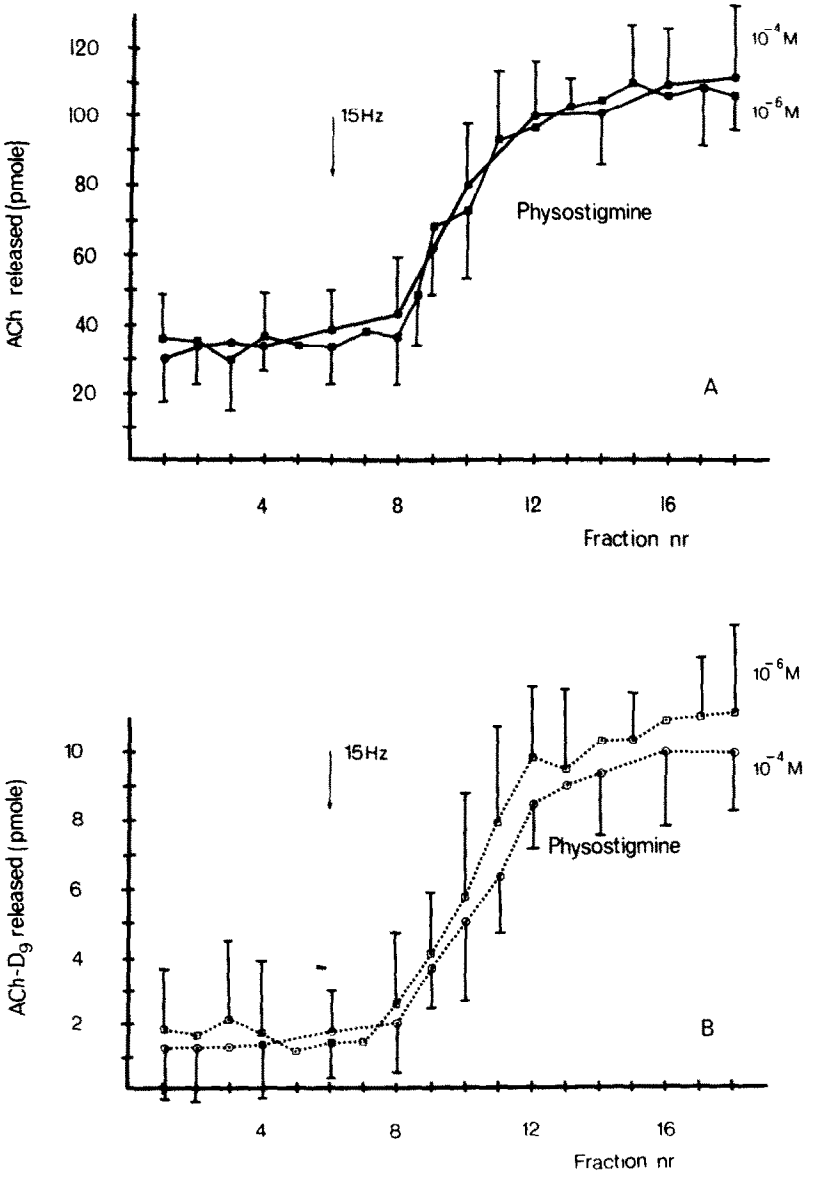

Fig. 3. Release of ACh into the perfusion fluid after pretreatment of the hemidiaphragm with different concentrations of physostigmine. After collection of fraction 6 the hemidiaphragm was stimulated indirectly through the phrenic nerve. Stimulation time 1 hr (corresponding to fractions 7–18). Ch-D_9 (10^{-6} M, deuterium labelled) was present in the perfusion fluid both during equilibration and throughout the experiment. Vertical bars indicate the $\pm t \cdot$SEM values. Value of t correspond to P = 0.05. See text for further details: (A) unlabelled ACh; (B) labelled ACh(D_9).

with varying concentrations of soman, DFP, Vx and sarin in the stimulated condition (15 Hz for 1 hr) on the same pools of ACh are shown in Table 2. The concentrations used are well above that necessary for cholinesterase inhibition. In the unstimulated condition values for endogenous ACh obtained for the organophosphate inhibitors seemed not to vary in the concentration range used and were similar to those obtained for physostigmine (Table 1). In the stimulated condition, however, treatment with increasing concentrations (10^{-6}–10^{-4} M) of soman, DFP and Vx increased the total amounts of unlabelled endogenous ACh and decreased the incorporation of the choline (D_9) isotope into the endogenous ACh pool. As shown in Table 2 these two effects combined caused a considerable reduction in the relative amounts of endogenous labelled ACh (D_9/D_0).

In contrast the experiments with sarin in the stimulated condition did not show any effects in either the endogenous level of ACh or in the incorporation of the choline isotope into ACh (Table 2). These observations also indicate that the interesting findings for soman, DFP and Vx are not due to the experimental conditions which were similar for the four inhibitors.

Table 2 also shows that hemicholinium (10^{-5} M) and soman present together in the perfusion fluid reduced the levels of both unlabelled and deuterium labelled endogenous ACh.

The spontaneous release of unlabelled ACh (10^{-5} M physostigmine) without stimulation was found to be about 6 pmole/min/hemidiaphragm (see Methods for experimental design). The corresponding release of labelled ACh was 0.2 pmole/min/hemidiaphragm. In the unstimulated condition the release was found to be maintained at this level throughout the experiment. In the stimulated condition (15 Hz for 1 hr corresponding to fraction 18, see Fig. 3) the release for unlabelled ACh was 22 pmole/min/hemidiaphragm and for labelled ACh 1.8 pmole/min/hemidiaphragm. The percentage of labelled to unlabelled ACh (D_9/D_0%) was therefore calculated to be about 3% for spontaneous and 8% for evoked release (Fig. 3). The values were not found to vary appreciably for physostigmine in the concentration range 10^{-6}–10^{-4} M.

The effects on release of pretreatment of the hemidiaphragm with varying concentrations of soman, DFP, Vx and sarin are shown in Table 3 and Figs 4 and 5. In the unstimulated condition release values for ACh were not found to vary appreciably for the inhibitor concentrations used (10^{-6}–10^{-4} M) and were fairly similar to those obtained for physostigmine (see also Fig. 3). During stimulation (15 Hz), however, pretreatment with high concentrations (10^{-5}–10^{-4} M) of soman, DFP and Vx decreased the release of unlabelled ACh in a dose dependent manner. At the same time reduction in the release of deuterium labelled ACh was found (Fig. 4). In Table 3 it is shown that the relative proportions of labelled to unlabelled ACh (D_9/D_0%) for evoked release decreased at increasing concentrations of the organophosphates soman, DFP and Vx.

In contrast pretreatment with high concentrations

Table 3. Values of released ACh from the unstimulated and stimulated hemidiaphragm (15 Hz) after treatment with organophosphates

Inhibitor conc. (M)	Soman			DFP			Vx			Sarin			Soman + HC-3 (10^{-5} M)		
	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%	D_0	D_9	D_9/D_0%
Unstimulated															
10^{-4}	6.0 ± 0.2	0.3 ± 0.1	5.0	6.8 ± 0.3	0.4 ± 0.2	5.9	5.4 ± 0.3	0.2 ± 0.1	3.7	6.6 ± 0.2	0.3 ± 0.1	4.5	—	—	—
10^{-5}	6.8 ± 0.4	0.3 ± 0.2	4.4	6.0 ± 0.2	0.3 ± 0.1	5.0	5.0 ± 0.2	0.3 ± 0.1	6.0	7.0 ± 0.3	0.3 ± 0.1	4.3	5.0 ± 0.2	0.2 ± 0.1	4.0
10^{-6}	7.4 ± 0.2	0.4 ± 0.1	5.4	—	—	—	6.4 ± 0.2	0.2 ± 0.1	3.1	6.8 ± 0.4	0.3 ± 0.2	4.4	—	—	—
15 Hz															
10^{-4}	6.6 ± 0.3*	0.4 ± 0.1*	6.1	8.4 ± 0.2	0.5 ± 0.1	6.0	6.4 ± 0.2*	0.3 ± 0.1*	4.7	14.0 ± 0.7	1.6 ± 0.2	11.4	—	—	—
10^{-5}	9.2 ± 0.4*	0.8 ± 0.1*	8.7	9.0 ± 0.3	0.9 ± 0.2	10.0	10.2 ± 0.3*	0.8 ± 0.2*	7.8	14.0 ± 0.5	1.5 ± 0.1	10.7	9.8 ± 0.2	0.3 ± 0.1	3.1
10^{-6}	17.6 ± 0.8	2.0 ± 0.1	11.4	—	—	—	17.0 ± 0.7	1.7 ± 0.1	10.0	15.6 ± 0.7	1.4 ± 0.1	9.0	—	—	—

Values based on perfusate fractions 3 and 18 corresponding to unstimulated condition and to 1 hr stimulation, respectively (see also Figs 3–6). The experiments refer to the continuous presence of 10^{-6} M Ch-D_9 in the perfusion fluid (in combination with Hc-3 when used). D_9 and D_0 refer to ACh with and without deuterated choline incorporated, respectively. The results are given in pmole ACh/min/hemidiaphragm and are based on at least four independent determinations. Values are expressed as mean ± SD. Asterisks indicate significant differences compared to values obtained for 10^{-6} M inhibitor concentration ($P = 0.05$) determined by the Student's t-test. For further details, see Methods.

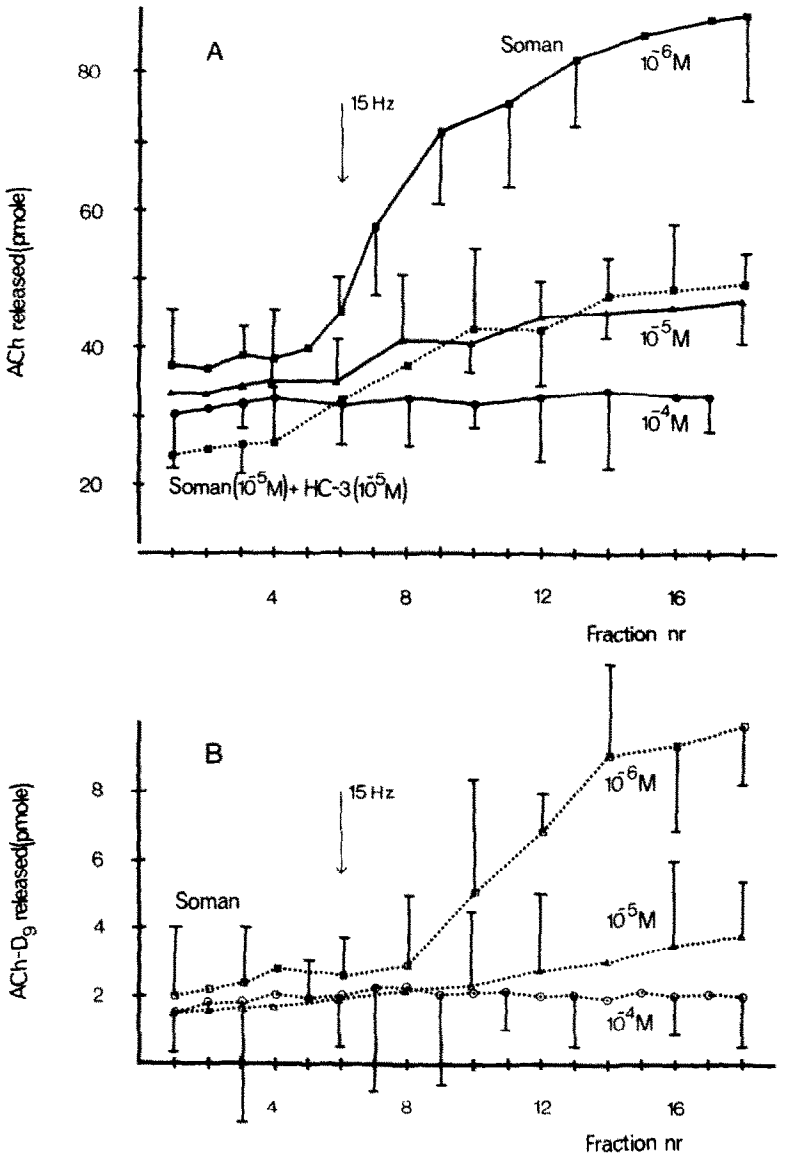

Fig. 4. Release of ACh into the perfusion fluid after pretreatment of the hemidiaphragm with different concentrations of soman. Results obtained for treatment with soman and hemicholinium in combination are also included. After collection of fraction 6 the hemidiaphragm was stimulated indirectly through the phrenic nerve. Stimulation time 1 hr (corresponding to fractions 7–18). Ch-D_9 (10^{-6} M, deuterium labelled) was present in the perfusion fluid both during equilibration and throughout the experiment. Vertical bars indicate the $\pm t \cdot$SEM values. Value of t correspond to $P = 0.05$. See text for further details: (A) unlabelled ACh; (B) labelled ACh (D_9).

of sarin and physostigmine did not cause decrease in release of unlabelled or labelled ACh (Table 3 and Figs 3 and 5).

The results given in Table 3 and Fig. 4 do not indicate changes in spontaneous and evoked release of unlabelled ACh for hemicholinium (10^{-5} M) and soman present together in the perfusion fluid compared to soman alone. Release of labelled ACh, however, seemed to be reduced in the stimulated condition.

Some experiments were also performed to get an indication of how fast ACh incorporated with choline-D_9 could be replaced by ACh incorporated with choline-D_{13} (by substitution of 10^{-6} M choline-D_9 with 10^{-6} M choline-D_{13} in the perfusion fluid). The actual values obtained are presented in Fig. 6 and indicate that during stimulation (15 Hz) and pretreatment with $3 \cdot 10^{-5}$ M soman the whole exchange process occurred in about 20 min.

DISCUSSION

The present paper describes a modification of the phrenic nerve–hemidiaphragm preparation from the rat. The preparation was kept in moist oxygen gas and perfused retrogradely from a phrenic vein. To ensure a proper flow resistance throughout the capillary bed a part of the body wall was left surrounding

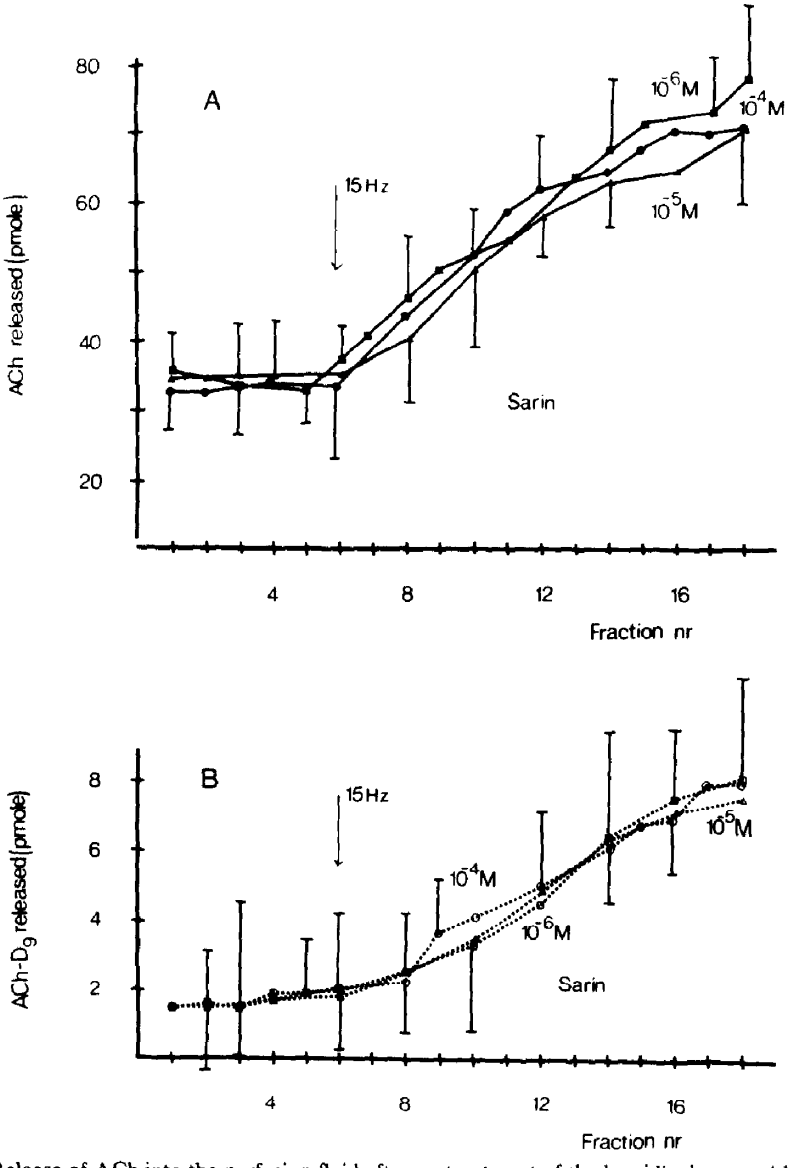

Fig. 5. Release of ACh into the perfusion fluid after pretreatment of the hemidiaphragm with different concentrations of sarin. After collection of fraction 6 the hemidiaphragm was stimulated indirectly through the phrenic nerve. Stimulation time 1 hr (corresponding to fractions 7–18). Ch-D_9 (10^{-6}M, deuterium labelled) was present in the perfusion fluid both during equilibration and throughout the experiment. Vertical bars indicate the $\pm t \cdot$SEM values. Values of t correspond to P = 0.05. See text for further details: (A) unlabelled ACh; (B) labelled ACh (D_9).

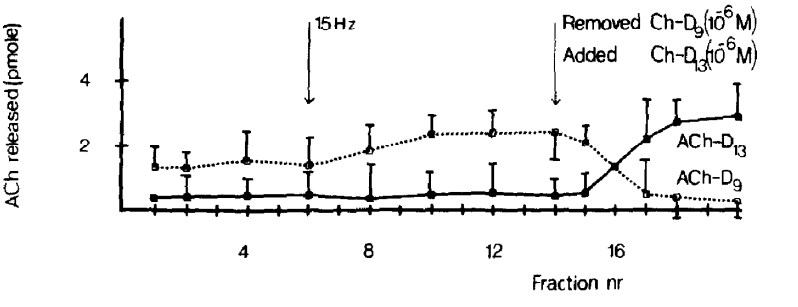

Fig. 6. ACh-D_9 and ACh-D_{13} isotopes released from the hemidiaphragm following replacement in the perfusion fluid of the Ch-D_9 by the Ch-D_{13} isotope. Stimulation time 1 hr (corresponding to fractions 7–18). Vertical bars indicate ±SEM values. Perfusion rate 0.2 ml/min and fraction size 1 ml indicate the whole exchange process to occur in about 20 min. Values refer to pretreatment with $3 \cdot 10^{-5}$ M soman.

the diaphragm muscle. Values for spontaneous and evoked synthesis and release of ACh in this preparation are compared and discussed in relation to results obtained with different modifications of hemidiaphragm preparations. For DFP, soman and Vx, different effects on different pools of ACh were observed. These effects could not be demonstrated with physostigmine and sarin. The effects were dose dependent and obtained at much higher concentrations than necessary for cholinesterase inhibition.

In their unperfused diaphragm Gundersen *et al.* [12, 13] report the value 1.5 pmole/mg tissue corresponding to about 150 pmole/hemidiaphragm for the ACh present endogenously in the unstimulated condition after physostigmine inhibition. This level of ACh seemed to be very stable for a long period of time (6 hr). Stimulation of the phrenic nerve increased the endogenous ACh content. Depending on the stimulation protocol values up to twice as much endogenous ACh could be observed [12, 13]. This is in agreement with our experiments also showing an increase in endogenous ACh following stimulation. For exposure to similar concentration of the choline isotope comparable incorporation into ACh was found (1.4% by [12, 13], 2.3% in our work). The ACh level, however, was generally found to be higher in our experiments.

Previously Bierkamper and Goldberg [5, 6, 9] used another modification of the vascularly perfused phrenic nerve-hemidiaphragm preparation to study choline incorporation and ACh release. They used a Hepes buffer perfusion fluid instead of a Tyrode solution as we used in our experiments. Bierkamper and Goldberg also report that they trimmed the ribs away from the diaphragm muscle which made it necessary to ligate major vessels cut during the trimming to prevent leakage. Bierkamper and Goldberg also used a lower frequency (7 Hz) for electric stimulation of the phrenic nerve than we used in our experiments (15 Hz). These authors found 0.95 and 7.75 pmole/min/hemidiaphragm for spontaneous and evoked (7 Hz) release, respectively, corresponding to 6.0 and 21.0 (15 Hz) pmole/min/hemidiaphragm in our experiments. Measurements by others for spontaneous release from diaphragms not vascularly perfused [3, 11–13, 28] ranging from 0.5 to 2.4 pmole/min/hemidiaphragm indicate that our value is unexpectedly high. According to Gundersen *et al.* [12, 13] and Bierkamper and Goldberg [6] the use of different concentrations of K^+, Mg^{2+} and Ca^{2+} ions in the various fluids used for organ baths and perfusion can be ruled out as an explanation for this. The high spontaneous ACh release in our experiments was not only observed after inhibition of the diaphragm AChE by DFP, but also by soman, Vx and sarin as well as by the carbamate physostigmine. Our results might, however, indicate that vascularly perfused hemidiaphragms both release and recover a higher proportion of ACh than unperfused preparations.

The present results clearly show the kinetics of the incorporation of exogenously supplied choline into endogenous as well as into released ACh. The data (Table 1) also show that stimulation increased preferentially the synthesis of labelled ACh in the endogenous and released pools. As a whole, however, the results show that ACh turnover during synaptic activity only involves a small part of the ACh store.

The results in our work, however, indicate a fast turnover of choline since the ACh-D_9 isotope released is replaced by the ACh-D_{13} isotope in 20 min. The low incorporation of external choline might indicate a large pool of choline present endogenously. This conclusion is in accordance with observations by Heilbronn (personal communication) (see also MacIntosh and Collier [17]).

The data in Table 1 seem to verify the notion of preferential release of newly-synthesized ACh during stimulation [3, 17]. Incorporated levels of labelled ACh are clearly shown to be higher in released than in endogenous ACh. The increase in incorporated radioactivity for higher stimulation frequencies is also more pronounced for released than for endogenous ACh.

Higher endogenous ACh levels, decreased incorporation of labelled choline into endogenous ACh and decreased ACh release were found in the stimulated hemidiaphragm after treatment with high concentrations (10^{-5} M and above) of soman, DFP and Vx. For sarin treatment, however, such alterations could not be observed.

The different effects caused by two closely related cholinesterase inhibitors like soman and sarin in high concentrations are surprising and interesting. It might be of importance that sarin is the most water soluble of these inhibitors. In agreement with the present finding (Fig. 3), the release was not affected by high concentrations of the carbamate physostigmine, which is another water soluble acetylcholinesterase inhibitor [6].

The reduction in release of ACh from the hemidiaphragm after treatment with high concentrations of soman, DFP and Vx might be due to a direct effect of these compounds on the release mechanism. Another possibility is that high concentrations of these inhibitors prevent electrical impulse transmission in the terminals of the phrenic nerve. The spontaneous release of ACh in the diaphragm does not seem to be affected as shown in Table 3. Reduced ACh release at high concentrations of the inhibitors occurs simultaneously with an increase in endogenous ACh. The Ch uptake mechanism seems to be intact, because competitive inhibition of Ch uptake by Hc-3 caused a decrease in endogenous ACh for soman inhibition (Table 2). The regulation of the endogenous ACh pool is, therefore, probably controlled by synthesis and release and not by the Ch uptake.

It might be possible that reduced ACh release and increase in endogenous ACh as shown in the present work are related to the formation of the so-called surplus ACh pool. Surplus ACh accumulates in the junctional region of a diaphragm whose acetylcholinesterase has been inactivated by the use of a lipid soluble cholinesterase inhibitor [17]. It should be mentioned, however, that the inhibitor physostigmine does not conform to this notion. The literature [3, 17] as well as data in the present work (Fig. 3 and Table 1) indicate that physostigmine still causes

accumulation of surplus ACh even if ACh release was not affected.

Data in Table 2 and 3 show that the increase in endogenous ACh during soman inhibition was accompanied by a decrease in labelled endogenous ACh and a decrease in release of ACh. Since the other experiments with physostigmine show that released ACh is newly synthesized ACh, the increase in endogenous ACh cannot simply be due to an accumulation of released ACh. During high concentration of soman the release of ACh and the synthesis of newly formed ACh (deuterium labelled) are obviously inhibited.

One of the main advantages of using vascular perfusion is the large reduction in fluid volumes compared to bath experiments. On this background the release of ACh to the perfusate after the onset of stimulation must be characterized as remarkably slow as shown in the present work. This could mean that ACh released from presynaptic nerve terminals were somehow trapped for a considerable length of time before being released into the perfusate. In studies of the effects of α-bungarotoxin on the neuromuscular transmission in the rat hemidiaphragm Miledi *et al.* [14] in fact suggest that normally some of the ACh is taken up by the muscle fibers. It might therefore be possible that the fat soluble inhibitors in high concentrations interferes with the release or leakage from these muscle fibers. Albuquerque *et al.* [29] have shown that cholinesterase inhibitors in high concentrations may have different effects on the post-synaptic acetylcholine receptor ionic channel complex thus indicating that such inhibitors also have effects in addition to their function as inhibitors of cholinesterase.

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