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SURPLUS ACETYLCHOLINE AND ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

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SUMMARY

1. Skeletal muscles from rat, mouse and frog were incubated under different conditions and the amounts of acetylcholine (ACh) extractable from the tissue and released into the medium were determined by mass fragmentography. In some experiments measurements were made of the amounts of ACh ('bound' ACh) surviving in a muscle homogenate to which an excess of acetylcholinesterase had been added. In other experiments the membrane potentials, end-plate potentials (e.p.p.s), and miniature end-plate potentials (m.e.p.p.s) were studied.

2. During incubation in Ringer medium the ACh content of the rat hemidiaphragm usually did not change, but after inhibition of cholinesterase by soman the ACh content rose gradually from about 100 to 150 pmol to a plateau of about 400 pmol after 4 h. A similar formation of 'surplus ACh' after cholinesterase inhibition was found in the mouse diaphragm, but not in the frog sartorius muscle.

3. Surplus ACh accumulated predominantly in the end-plate region of the rat diaphragm. In muscles, 16–18 h after *in vivo* denervation, the capacity to form surplus ACh was decreased by more than 80%.

4. The amount of ACh diffusing from the resting hemidiaphragm into the incubation medium ('resting release') varied between 0·5 and 0·9 pmol min^{-1} in different experiments; it remained at the same level during accumulation of surplus ACh. It was reduced by more than 80% 16–18 h after denervation.

5. The amplitude of m.e.p.p.s and e.p.p.s did not increase while surplus ACh was accumulating.

6. Incubation of hemidiaphragms in Ringer solution containing [^{3}H]choline caused the formation of [^{3}H]ACh. Additional amounts of [^{3}H]choline were incorporated into ACh when the nerve was stimulated for 60 min. However, incubation in the presence of soman (3,3-dimethyl-2-butylmethylphosphonofluoridate), in the absence of stimulation, did not cause an increase of the [^{3}H]ACh content of the muscles.

7. From hemidiaphragms with active cholinesterase about 120 pmol ACh was lost after prolonged nerve stimulation or incubation with 50 mM-KCl in the presence of hemicholinium-3, and about 35 pmol remained in the tissue. In soman-treated

muscles, containing surplus ACh, about as much ACh was released by nervous stimulation as from untreated hemidiaphragms, and much more ACh remained unreleased.

8. Transection of the muscle at both sides of the end-plate or incubation of intact muscles in the presence of 50 mM-KCl depolarized the muscle fibres to −35 and −31 mV, respectively. Surplus ACh was partially released by 50 mM-KCl, but not by muscle transection.

9. The high-[K^+]-evoked release of surplus ACh was Ca^{2+} dependent.

10. Incubation of soman-treated muscles in the presence of 10^{-7} M-[^{3}H]ACh resulted in the uptake of only a few picomoles of [^{3}H]ACh by the tissue.

11. 'Bound' (presumably vesicular) ACh was not increased after accumulation of surplus ACh.

12. It is concluded that both the bulk of surplus ACh and the ACh which is released by nerve stimulation are synthesized in the nerve ending, but in *different* compartments, that surplus ACh is localized in a cytoplasmic compartment of the nerve ending, or of the axon close to the ending, and that this compartment does not easily exchange ACh with the compartment from which the ACh quanta originate. The possibility is discussed that the choline used for the synthesis of surplus ACh may be recruited from an intraneuronal choline pool.

INTRODUCTION

In many studies pertaining to the synthesis, storage and release of acetylcholine (ACh) in skeletal muscle, an anticholinesterase is used to prevent the hydrolysis of released ACh. In the rat diaphragm (Potter, 1970; Doležal & Tuček, 1983) and in several other organs (see MacIntosh & Collier, 1976) cholinesterase inhibition not only leads to the preservation of released ACh but also to a gradual, often considerable, rise of the ACh content in the tissue. The extra amount of ACh accumulating after cholinesterase inhibition has been called 'surplus ACh' (Birks & MacIntosh, 1961), to be distinguished from the ACh store ('depot ACh') normally involved in synaptic transmission.

From skeletal muscle *in vitro* treated with an anticholinesterase, ACh diffuses continuously into the incubation medium, even when the preparation is not stimulated. This 'resting release' presumably takes place by a non-quantal release process (Straughan, 1960; Mitchell & Silver, 1963; Fletcher & Forrester, 1975; Miledi, Molenaar & Polak, 1977; Katz & Miledi, 1977, 1981; Gorio, Hurlbut & Ceccarelli, 1978).

Although surplus ACh and resting release of ACh do not appear to be intimately connected with the neurotransmitter function of ACh, they might give some clues about the mechanisms of synthesis and storage of the transmitter. Moreover, surplus ACh and resting release of ACh account for such an important proportion of the total turn-over of ACh, that they cannot be ignored in any study of its compartmentation.

In the present experiments on the rat diaphragm we attempted to analyse the origin of surplus ACh and resting release of ACh by studying, among other things, the way they were influenced by denervation. It is concluded that the greater part of surplus ACh is synthesized and stored in the motor nerve endings, and that these

are the main source of the resting release of ACh. Surplus ACh did not seem to be available for release by nervous impulses, but it was partially released by a high KCl concentration in the medium.

METHODS

Hemidiaphragm from rats

Hemidiaphragms from 5–6-week-old small male Wistar (WAG/Rij) rats weighing 70–80 g were used. In some experiments about 2 cm of phrenic nerve was left attached to the muscle for electrical stimulation; in other experiments the nerve was cut close to the muscle. When the effect of denervation was studied, the rat was anaesthetized with ether and the left phrenic nerve was cut about 6 mm above its entrance into the muscle (Miledi & Slater, 1970), 16–18 h before the diaphragm was isolated. The right hemidiaphragm was used as innervated control preparation. The hemidiaphragm was kept in oxygenated (95 % O_2 and 5 % CO_2) Ringer medium at 30 °C (in a few experiments 22 °C) of the following composition (mM): NaCl, 114; KCl, 4·6; $NaHCO_3$, 25; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 11. In 'Ca^{2+}-free' medium the $CaCl_2$ was omitted, the $MgCl_2$ concentration increased to 2 mM and 0·5 mM-ethyleneglycol-bis-(β-aminoethylether) *N,N'*-tetraacetic acid (EGTA) added. To exclude ACh release from intercostal muscle the hemidiaphragm was dissected from the ribs before incubation. It should be noted that this procedure, even if done with care, invariably damaged the muscle fibre ends. However, this did not appreciably influence the ACh content of the preparation either before or after incubation (R. L. Polak, unpublished observations). The membrane potential at the end-plate, $-64 \pm 2{\cdot}1$ mV (mean ± S.E. of mean) in seventy-seven muscle fibres from eight rat hemidiaphragms with ribs attached, was reduced to $-56 \pm 2{\cdot}0$ mV in 103 muscle fibres from ten hemidiaphragms after 10–120 min incubation following removal of the ribs ($P_2 < 0{\cdot}05$, Welch's *t* test). For complete and irreversible inactivation of cholinesterase the hemidiaphragms were incubated for 15 min in the presence of 0·2 μM-soman. In some experiments higher concentrations of soman were applied for longer periods, but this was found to be unnecessary in later experiments. In other experiments, different cholinesterase inhibitors were used.

For electrical stimulation the phrenic nerve was placed over bipolar platinum electrodes. Supramaximal pulses of 100 μs were applied either at 9 or at 44 Hz in 0·2 s trains, one train per second. For prevention of anoxia due to energy expenditure by muscle contractions, the preparation was incubated for 90 min in medium containing α-bungarotoxin (5 μg/ml) before being subjected to prolonged nerve stimulation.

Skeletal muscle from mice and frogs

Hemidiaphragms from male and female mice weighing 20–30 g were incubated at 22 °C in medium of the same composition as used for the rat hemidiaphragm.

Sartorius muscles of *Rana temporaria* were isolated and incubated at 22 °C, as described by Miledi, Molenaar & Polak (1980). Ringer solution of the following composition (mM) was used: NaCl, 116; KCl, 2; $CaCl_2$, 1·8; sodium phosphate, 2 (pH 7·2). For cholinesterase inhibition 100 μM-diethyl-dimethylpyrophosphonate (DEPP) was present in the medium.

Transection of muscle fibres

The muscle fibres were cut on both sides of the row of end-plates at a distance of 2–3 mm from them, and the three segments were incubated together.

Uptake of [^{3}H]ACh

Rat hemidiaphragms were incubated for 30 min in the presence of 10 μM-soman (3,3-dimethyl-2-butylmethylphosphonofluoridate). Subsequently, they were incubated for 60 min at 22 °C in 5 ml Ringer solution containing [^{3}H-acetyl]choline (20000 ct/(min ml), *ca.* 10^{-7} M), and for an additional 60 min period in renewed medium of the same composition. At $t = 120$ min the muscles were gently blotted with Kleenex paper and the ribs were removed from the diaphragm. The muscles were then soaked in Ringer solution for 5 min ('wash') and subsequently homogenized in 10 % (w/v) trichloroacetic acid (TCA). Labelled acetic acid and ACh in the media, the 'wash' fluid and the extracts were purified and measured by liquid scintillation counting according to procedures earlier

described (Miledi *et al.* 1980). The samples were counted, and recounted after addition of standard amounts of labelled ACh in order to correct for variations in counting efficiency.

Electrophysiology

Membrane potential, miniature end-plate potentials (m.e.p.p.s) and end-plate potentials (e.p.p.s) in the rat hemidiaphragm were recorded by conventional techniques. The amplitude and frequency of the m.e.p.p.s were measured directly on the screen of a storage oscilloscope.

Homogenization of muscle

Muscles were disrupted during 0·5 min with an Ultra-Turrax homogenizer either at 20 °C in 3 ml acetonitrile containing 2 % (w/v) TCA and ACh-d_{16} as internal standard for the determination of endogenous ACh ('total ACh') or in 4 ml Ca^{2+}-free Ringer solution at 0 °C. The first procedure extracts all ACh from the tissue (Miledi, Molenaar & Polak, 1977, 1980). The homogenate in Ca^{2+}-free Ringer solution to which ACh-d_9 was added, was incubated for 4·5 min at 0 °C in the presence of 5 μg/ml of electric eel acetylcholinesterase, which hydrolysed all ACh in free solution as judged by the hydrolysis of the added ACh-d_9. Thereafter TCA (final concentration 10 %, w/v) was added for deproteination and ACh-d_{16} as an internal standard for the determination of endogenous ACh ('bound ACh') which had survived the action of electric eel acetylcholinesterase. This procedure was essentially that of Miledi, Molenaar & Polak (1982).

Synthesis of [^{3}H]ACh from [^{3}H]choline

Rat hemidiaphragms, from which the ribs had been removed, were incubated for 45 min in the presence of α-bungarotoxin (5 μg/ml). Subsequently, they were incubated for 60 min at 26–27 °C in 5 ml Ringer solution containing [^{3}H]choline (5 μCi/ml, 0·15 mM), washed for 15 min and then homogenized in 2 % (w/v) TCA in acetonitrile. Small samples were withdrawn from the incubation media and the crude homogenates for the estimation of their total ^{3}H contents. The [^{3}H]ACh was purified after addition of [^{14}C-acetyl]choline as an internal standard, by repeated precipitation with potassium periodide as described below for endogenous ACh, with the exception that each sample was divided into two portions immediately before the purification procedure. One portion was treated with acetylcholinesterase in order to destroy all [^{3}H]ACh and the amount of ^{3}H measured after the purification procedure was used to correct the amount of [^{3}H]ACh in the other portion for traces of [^{3}H]choline which had not been removed by the purification procedure. All samples were counted in two channels (^{3}H and ^{14}C) of a liquid scintillation counter.

Determination of ACh

ACh was measured by pyrolysis–mass fragmentography on a micropacked column (Polak & Molenaar, 1979) after extraction and purification as described by Miledi *et al.* (1980). The purification method consisted of extraction and deproteination with 2 % TCA (w/v) in acetonitrile followed by four successive precipitations with potassium periodide (Welsch, Schmidt & Dettbarn, 1972; Polak & Molenaar, 1974). Samples of ACh in homogenates made in Ringer solution, were purified as described for ACh in incubation media (Miledi *et al.* 1980).

Materials

Soman and DEPP were kindly provided by Dr H. P. Benschop, Prins Maurits Laboratory TNO, Rijswijk, The Netherlands. Tetrodotoxin was from Boehringer Mannheim; fully deuterated acetylcholine chloride (ACh-d_{16}) was from Merck, Sharp and Dohme; [^{3}H-acetyl]choline chloride (250 mCi/mmol) was from Amersham; [*N*-methyl^{3}H]choline (36 Ci/mmol) from New England Nuclear; hemicholinium-3 (HC-3), twice recrystallized from ethanol before use, was from Aldrich; d-tubocurarine chloride was from Fluka; α-bungarotoxin and electric eel acetylcholinesterase (350 u/mg protein) were from Sigma.

RESULTS

Surplus ACh in rat hemidiaphragms

When rat hemidiaphragms were incubated under resting conditions without an anticholinesterase, their ACh content usually remained at an initial level of about 100 pmol (Fig. 1), but in a few experiments the mean ACh content gradually increased

and reached levels which were about 1·5 times the original value. In hemidiaphragms treated with the anticholinesterase soman, the ACh content rose always rapidly to much higher values than in untreated hemidiaphragms, and reached a plateau after about 150 min. Whereas the values found within a series of experiments were reproducible, those found in different series of experiments sometimes varied greatly, possibly due to seasonal effects or to changes in food composition. The rise in ACh content did not appear to be greatly influenced by the temperature of incubation.

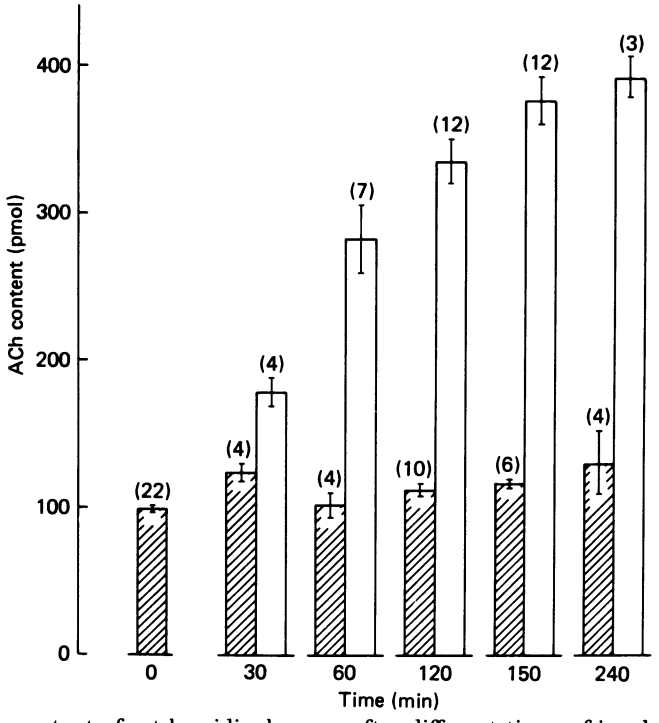

Fig. 1. ACh content of rat hemidiaphragms after different times of incubation at 30 °C. The columns represent ACh contents in pmol/hemidiaphragm: hatched columns, no anticholinesterase in medium; open columns, 0·2 μM-soman was present in the medium during the first 15 min of incubation. When incubation lasted 240 min, the soman was present in the medium (which was replaced at 60 min intervals) during the whole incubation period. Means ±S.E. of mean with numbers of observations in parentheses.

As shown in Fig. 2, most surplus ACh accumulated in the regions of the muscle where the end-plates are localized, suggesting that the nervous elements were involved in the formation of the main part of surplus ACh. However, the ACh content of end-plate-free regions often also increased.

In some experiments we added tetrodotoxin (0·5 μg/ml) to the medium to prevent the spontaneous nervous activity which may occur after cholinesterase inhibition (Masland & Wigton, 1940). This did not influence the accumulation of surplus ACh (not illustrated). In other experiments blockade of the nicotinic receptors by pre-incubation for 90 min with α-bungarotoxin did not influence the accumulation of surplus ACh; muscarinic ACh receptors were not involved, since atropine did not influence the formation of surplus ACh. Tabun, a different organophosphorus

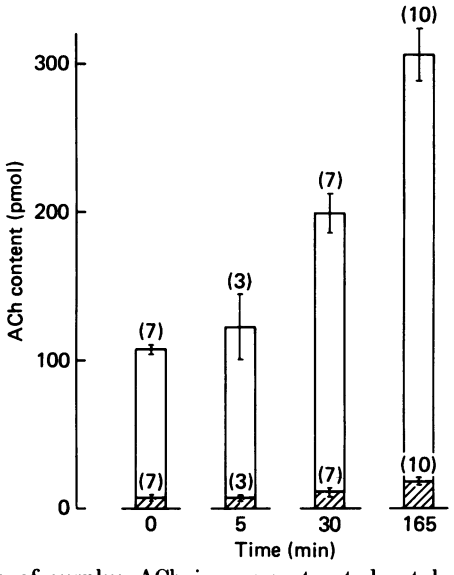

Fig. 2. Formation of surplus ACh in soman-treated rat hemidiaphragms. Rat hemidiaphragms were incubated at room temperature (22 °C). Soman (10 μM) was present in the medium during the first 5 or 30 min of incubation. After the incubation the hemidiaphragms were divided into segments containing nervous elements (open columns) and nerve-free segments (about 33 % of total mass, hatched columns). The columns represent ACh contents in pmol/hemidiaphragm (means ±S.E. of mean with numbers of observations in parentheses). ACh content in both nerve-containing and nerve-free regions increased statistically significantly with time (Student's t test, $P_2 < 0{\cdot}05$).

TABLE 1. Absence of surplus-ACh formation in the sartorius muscle of the frog

Time of incubation (h)	Total extractable ACh (pmol/muscle) Control a	DEPP p	(p/a) ×100
1	38±4·1 (6)	41±4·6 (6)	108±3·1 (6)
3	47±0·5 (2)	48±6·0 (2)	102±13 (2)
6	46±5·3 (5)	49±4·7 (5)	108±7·0 (5)

Frog sartorius muscles were extracted for determination of their ACh content after 1, 3 or 6 h incubation in the absence (a) or presence (p) of the anticholinesterase DEPP (100 μM). Means ±S.E. of mean with numbers of observations in parentheses.

inhibitor of cholinesterase, also caused the accumulation of surplus ACh, and so did neostigmine, a cholinesterase inhibitor of the carbamate ester type.

Mouse hemidiaphragm

In an experiment in which mouse hemidiaphragms were incubated at 22 °C, the ACh content was 77±10·9 ($n = 4$) pmol at 15 min, and 175±15·6 ($n = 4$) pmol at 120 min after the addition of soman (final concentration 2 μM) to the medium.

Frog sartorius muscle

As shown in Table 1 no surplus ACh accumulated in frog sartorius muscle incubated in the presence of DEPP which is an effective inhibitor of cholinesterase in frog muscle (Miledi *et al.* 1980).

Resting release of ACh from the rat hemidiaphragm

No ACh could be detected in media in which hemidiaphragms with active cholinesterase were incubated. However, after cholinesterase inhibition there was a

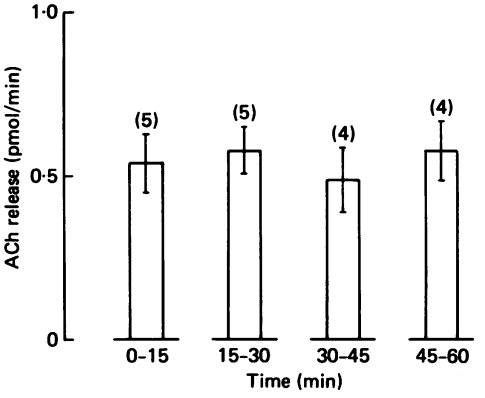

Fig. 3. Resting release of ACh during accumulation of surplus ACh in rat hemidiaphragms. The hemidiaphragms were pre-incubated for 90 min with α-bungarotoxin (5 μg/ml). Thereafter the hemidiaphragms were rinsed and either extracted immediately ($t = 0$) for determination of ACh content or after an additional 60 min period of incubation in medium containing 1 μM-soman ($t = 60$ min). The medium was replaced at 15 min intervals and the ACh released into the medium was determined. The ACh content of the hemidiaphragms was $128 \pm 9{\cdot}9$ (4) pmol at $t = 0$ min and 286 ± 32 (5) pmol at $t = 60$ min. The columns represent the amounts of ACh released (pmol/min, means ±S.E. of mean, with numbers of observations in parentheses).

continuous overflow ('resting release') of ACh from the rat hemidiaphragm into the incubation medium, at a mean rate varying between 0·5 and 0·9 pmol/min in different experiments. As shown in Fig. 3, the rate of resting release of ACh remained at the same level during a 60 min period of incubation, in which about 160 pmol surplus ACh was accumulating. This suggests that either the ACh released under resting conditions does not derive from the surplus ACh pool or that it is released from this pool by a process different from simple diffusion.

Effects of denervation on surplus ACh and resting release of ACh

The motor nerve endings in the rat diaphragm degenerate within 16 h after section of the phrenic nerve whereas the motor axons remain intact and able to conduct impulses for more than 24 h after denervation (Miledi & Slater, 1970). In order to test whether the motor nerve endings are involved in the formation of surplus ACh and in the resting release of ACh, we studied how these processes were affected by denervation.

As shown in Fig. 4, both the ACh content of hemidiaphragms immediately after isolation and the amounts of ACh accumulating after cholinesterase inhibition were reduced by more than 80% in hemidiaphragms isolated 16–18 h after section of the phrenic nerve, and the resting release of ACh was reduced by a similar proportion. Apparently the motor nerve endings contained the bulk of the ACh present in freshly dissected hemidiaphragms and were responsible for the synthesis of the greater part of surplus ACh and resting ACh release.

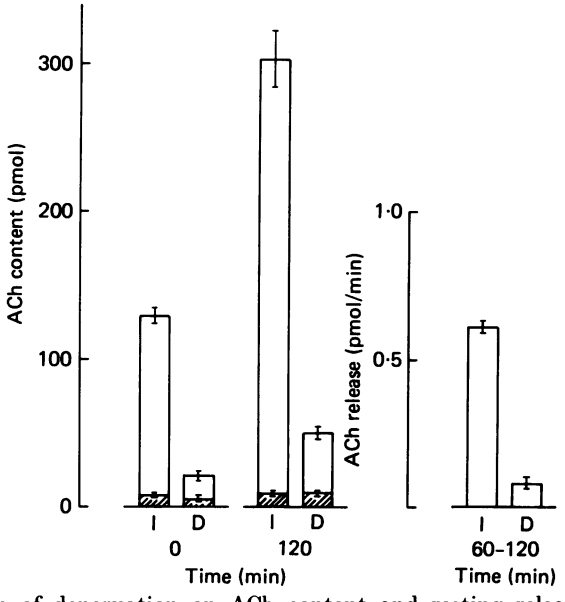

Fig. 4. Influence of denervation on ACh content and resting release of ACh in rat hemidiaphragms. The hemidiaphragms were extracted for determination of ACh content either immediately or after 120 min incubation at 30 °C. During the first 45 min of incubation 1 μM-soman was present in the medium. Thereafter the hemidiaphragms were rinsed three times with medium without soman. The medium contained tetrodotoxin (1·6 μM) throughout the incubation. The ACh released into the media between $t = 60$ and 120 min was measured. I, innervated; D, 16–18 h after section of phrenic nerve. Before extraction the hemidiaphragms were divided into segments containing nervous elements (open columns) and nerve-free segments (hatched columns), see left part of the Figure. Means ± S.E. of mean of six observations.

The effects of cholinesterase inhibition on the m.e.p.p.s and e.p.p.s

Cholinesterase inhibition by neostigmine caused an immediate increase of both decay time and amplitude of m.e.p.p.s, but the effect on amplitude was variable and not always observed (Fig. 5*A*). The m.e.p.p. size did not increase further on prolonged incubation in the presence of neostigmine. Incubation with soman led to the same result. When added to a medium containing neostigmine soman had no additional effect.

The decay time of e.p.p.s, induced by electrical stimulation of the phrenic nerve at a frequency of 5 Hz, in the presence of 0·5 μM-d-tubocurarine to prevent muscle contraction, increased after the addition of soman, whereas the amplitude, after a

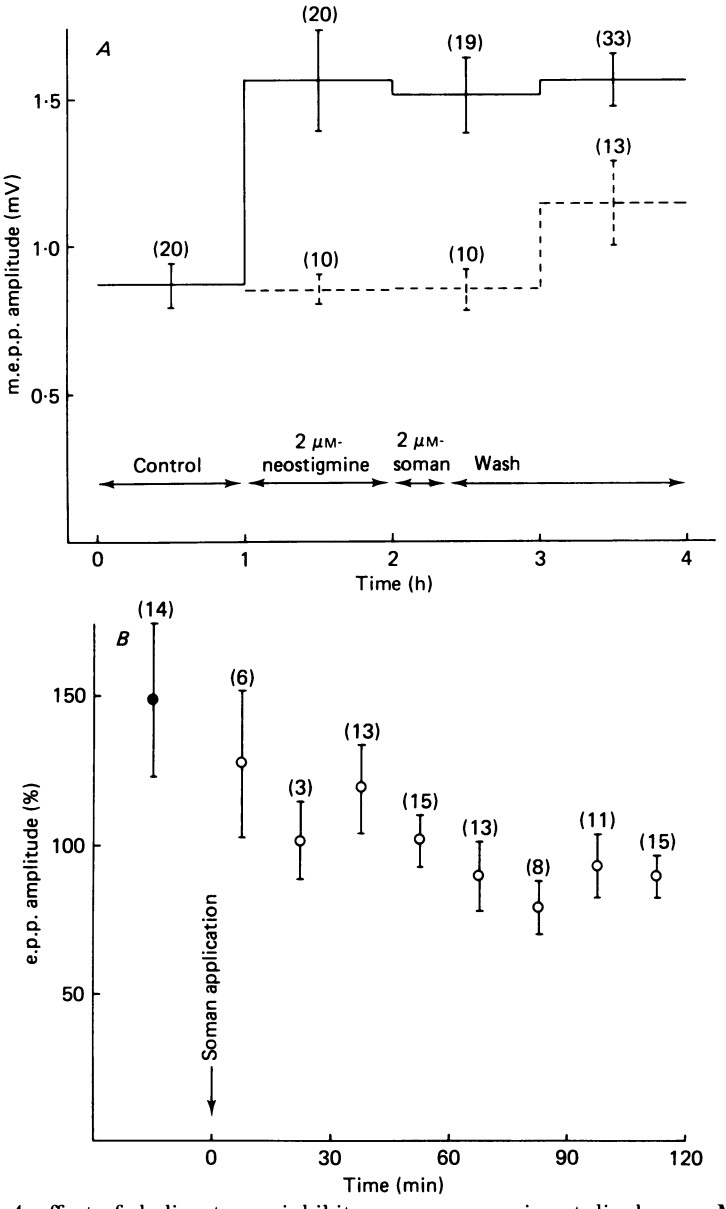

Fig. 5. *A*, effect of cholinesterase inhibitors on m.e.p.p.s in rat diaphragm. Muscles were incubated for 1 h in the presence of neostigmine, then washed with Ringer solution and treated for 20 min with soman (continuous line). The interrupted line refers to an experiment in which neostigmine was omitted. Tetrodotoxin (1·6 μM) was present to prevent spontaneous contractions of muscle fibres. The mean resting potential of the fibres was -63 ± 7 mV; the amplitude of m.e.p.p.s was normalized to -65 mV assuming an equilibrium potential of 0 mV. The temperature was 27–30 °C. *B*, e.p.p amplitude in rat diaphragm before (filled circles) and after cholinesterase inhibition by 2 μM-soman (open circles). The medium contained 0·5 μM-d-tubocurarine in order to prevent neuromuscular transmission during the continuous stimulation of the phrenic nerve at 5 Hz. The temperature was 25–30 °C. Because the e.p.p.s varied in size between different muscles, the amplitudes were normalized by being expressed as percentages of the mean amplitude of all e.p.p.s observed after soman in the different end-plates of one muscle. Means ± S.E. of mean. The figures above the points represent the numbers of end-plates tested in two to five rat muscles, except the interrupted line in *A* which represents data obtained in one muscle.

transient increase lasting less than 5 min, decreased by about 30 % (Fig. 5*B*), possibly due to a decrease of available transmitter or to a reversible post-synaptic blocking action resembling that of diisopropylfluorophosphonate (cf. van der Meer & Meeter, 1956; Kuba, Albuquerque & Barnard, 1973). The e.p.p. amplitude decreased a little further during the following 120 min of incubation. This reduction could be due to desensitization of ACh receptors caused by the prolonged effect of ACh at the end-plate (Meeter, 1969).

These results indicate that there was no increase of either quantal size or quantal content during accumulation of surplus ACh.

Effect of stimulation on ACh content

Subsequently we attempted to find out whether stimulus-evoked ACh release may recruit ACh from the surplus compartment. To this end we inhibited the synthesis of ACh by HC-3 after allowing 120 min of surplus ACh formation under the influence of soman, and then studied the decrease of the ACh content of the muscles, brought about by prolonged stimulation. In control experiments, muscles were incubated in the same way, but without soman in the first 120 min. As illustrated in Fig. 6, hemidiaphragms not treated with an anticholinesterase contained 157 pmol ACh and soman-treated muscles 334 pmol ACh after 120 min incubation. When the incubation was continued for an additional 60 min period, this time in the presence of HC-3, the ACh content decreased, probably due to the resting release of ACh. For unknown reasons, the loss of ACh was always greater in soman-treated than in untreated hemidiaphragms. In muscles with active cholinesterase nerve stimulation or incubation in a medium containing 50 mM-KCl resulted in a decrease of the extractable ACh content from 157 to 36 and 34 pmol, respectively. Since similar values were found also after 30 min stimulation in the presence of HC-3 in other experiments (not illustrated), the released ACh probably represented the total store of depot ACh, localized in the motor nerve endings, whereas the remaining ACh ('stationary' ACh, Birks & MacIntosh, 1961) was probably unavailable for release because it was localized mainly in the muscle fibres (see Discussion). In soman-treated hemidiaphragms, nerve stimulation caused a similar reduction of the ACh content as in untreated muscles, that is by an amount equivalent to the depot ACh. Consequently, as much as 194 pmol ACh remained unreleased. This was probably not due to inefficient stimulation of the nerve terminals, or damage to the nerve fibres, since in other experiments more than 300 pmol was released into the medium when the nerve was stimulated for 60 min in the absence of HC-3.

Although we have no absolute proof about its identity, we believe that the unreleasable ACh in soman-treated hemidiaphragms consisted of surplus plus stationary ACh. This would imply that little or no surplus ACh was available for release by nervous impulses. On the other hand, incubation in a high-[K^+] medium resulted in a much greater loss of ACh (243 pmol) from soman-treated hemidiaphragms containing surplus ACh than from untreated hemidiaphragms (157 pmol). Apparently, high-[K^+] medium caused the release of surplus ACh as well as depot ACh. Yet, more ACh (91 pmol) was present in the tissue afterwards than in hemidiaphragms not treated with an anticholinesterase, indicating that *not all* surplus ACh had been released.

In other experiments (not illustrated) it was found that the high-[K^+]-evoked release of the ACh from soman-treated and untreated hemidiaphragms was prevented completely in Ca^{2+}-free media; containing 2 mM-$MgCl_2$ and 0·5 mM-EGTA. Apparently, the high-[K^+]-evoked release of both depot and surplus ACh was Ca^{2+}-dependent.

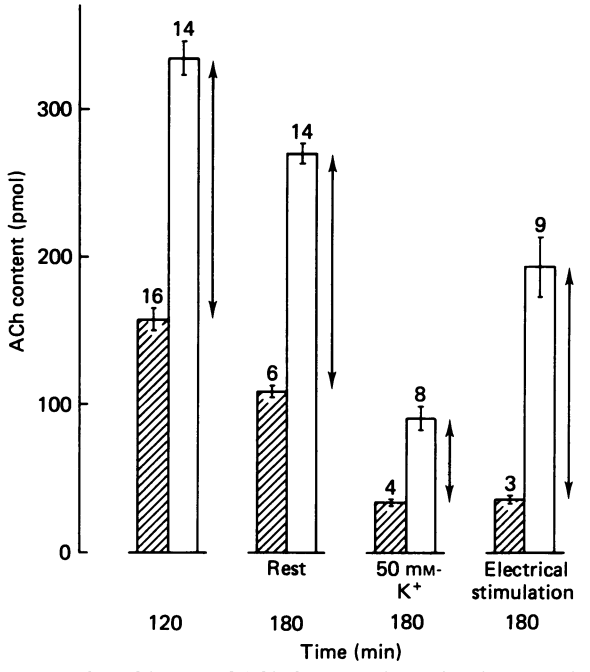

Fig. 6. Stimulation-induced losses of ACh from rat hemidiaphragms during incubation in the presence of HC-3. The muscles were incubated for 120 min in Ringer solution which did (open columns) or did not (hatched columns) contain 0·2 μM-soman between 0 and 15 min. Subsequently, the muscles were either extracted or incubated for an additional 60 min period during which 10 μM-HC-3 was present in the medium. During this period the medium contained either 50 μM-KCl, or the phrenic nerve was stimulated electrically (100 μs supramaximal pulses, 44 Hz in trains of 200 ms, one train per second) or the preparation was not stimulated (rest). In the experiments in which the phrenic nerve was stimulated and in the corresponding 120 min control experiments, α-bungarotoxin (5 μg/ml) was present in the medium between 0 and 90 min. Means ±S.E. of mean with number of observations in parentheses. The two-headed arrows indicate the amounts of surplus ACh present in the soman-treated hemidiaphragms.

Incorporation of labelled choline into ACh

Subsequently, we attempted to label the surplus ACh selectively by incubating muscles in the presence of labelled choline and soman, in the hope to obtain further information on the release of surplus ACh by stimulation. However, these experiments were unsuccessful, since we did not succeed in labelling the surplus ACh. The results in Table 2 show that the amount of [^{3}H]ACh synthesized and contained in the muscle after 60 min of labelling under resting conditions did not increase when the cholinesterase was inhibited by soman, a procedure which in other experiments (cf. Fig. 1) caused a more than twofold increase of the ACh content of the muscle.

Apparently, the choline in the interstitial space, trace labelled with [^{3}H]choline, had practically no access to the compartment in which surplus ACh was synthesized. On the other hand, external choline was readily incorporated into ACh under the influence of nervous stimulation (see Table 2). The actual amount of [^{3}H]ACh synthesized is unknown, but it must have been greater than the amount found in the tissue, since a part of it was probably released by the stimulation immediately after its formation. Table 2 further shows that the amounts of total ^{3}H in the preparations were similar under all experimental conditions.

TABLE 2. Acetylation of [^{3}H]choline in the rat hemidiaphragm

	[^{3}H]ACh ((ct/min) × 10^{-3})	Total ^{3}H ((ct/min) × 10^{-3})
Without soman	1·6 ± 0·31 (7)	370 ± 47 (7)
Soman	1·5 ± 0·24 (6)	290 ± 30 (6)
Without soman, nervous stimulation	3·2 ± 0·40 (6)*	290 ± 14 (6)
Soman	1·3 ± 0·36 (5)*	320 ± 31 (5)

The muscles were incubated for 45 min with α-bungarotoxin (5 μg/ml) and then for 60 min in medium containing 0·15 μM-[^{3}H]choline. During this period the nerve was stimulated electrically (0·2 s trains of 44 Hz, 1 train per second) in some experiments. The muscles were washed for 15 min in Ringer solution before being extracted for determination of their [^{3}H]ACh and total ^{3}H content. Mean values ± S.E. of mean with number of observations in parentheses.

* Statistically significant difference (Student's t test; $P_2 < 0·01$).

The effect of transection of the muscle fibres

The conclusion that the motor nerve endings are the site where most surplus ACh is synthesized, does not necessarily imply that surplus ACh is localized inside them. It is possible that the accumulation of surplus ACh results from the uptake of ACh, released from motor nerve endings, into other structures such as the muscle fibres. In that case it would become understandable why surplus ACh would be unavailable for release by nervous impulses in α-bungarotoxin-immobilized hemidiaphragms, whereas it was released in high-[K^+] media. Under the latter condition not only the motor nerve endings, but also the muscle fibres and Schwann cells are depolarized, and this might facilitate a possible efflux of ACh cations via the cell membrane. If surplus ACh is localized in the muscle fibres and released from them by depolarization in high-[K^+] medium, it should also be released if the muscle fibres are depolarized selectively by transection. This was tested in the experiments described below.

As shown in Table 3, the membrane of the muscle fibres at the end-plates was depolarized from −62 to −35 mV when the fibres were cut, and from −66 to −31 mV by the presence of 50 mM-KCl in the medium. Apparently the muscle fibres were depolarized to about the same extent under the two conditions. In addition the Table demonstrates that transection of the muscle fibres had some effect on the motor nerve endings: it caused a, partially transient, rise in the frequency of the m.e.p.p.s, notwithstanding the fact that the nerve branches had not been cut.

In the experiments of Fig. 7, the formation of surplus ACh in soman-treated hemidiaphragms was allowed to proceed for 120 min, and the incubation was then continued for another 60 min period under different conditions. When HC-3 was

present between 120 and 180 min, the formation of surplus ACh stopped and the ACh content of intact muscles decreased due to the resting release of ACh. Hemidiaphragms cut after 120 min lost statistically significantly more ACh than intact preparations, but the difference was small, and can be explained by the increased quantal release demonstrated in Table 3. The decrease in ACh content was the same in the absence and presence of HC-3, and the lost amount of ACh equalled the amount

TABLE 3. Membrane potential at end-plates during incubation in the presence of 50 mM-KCl or after transection of the muscle fibres at both sides of the row of end-plates and frequency of m.e.p.p.s at different intervals after transection

	Membrane potential (mV)	Number of end-plates
Control	$-66 \pm 1{\cdot}5$	46
50 mM-KCl	$-31 \pm 2{\cdot}7$	43
Control	$-62 \pm 2{\cdot}2$	70
Cut muscle	$-35 \pm 1{\cdot}8$	63
	m.e.p.p. frequency (/s)	Number of end-plates
Control before transection	$1{\cdot}2 \pm 0{\cdot}17$	70
0–10 min after transection	$37 \pm 1{\cdot}4$	10
10–20 min after transection	$13 \pm 3{\cdot}4$	20
20–30 min after transection	$13 \pm 6{\cdot}1$	30

Mean values $\pm$S.E. of mean from four muscles (50 mM-KCl) and seven muscles (transection).

recovered in the medium, indicating that no appreciable synthesis of ACh took place, even in the absence of HC-3. It is possible that the resting synthesis was inhibited because the transection of the muscle fibres in some way may have influenced the motor nerve endings. Just as shown above in Fig. 6 the incubation in a medium containing 50 mM-KCl caused a strong decrease of the ACh content of the tissue (Fig. 7). In the presence of HC-3 the decrease matched the overflow of ACh into the medium, demonstrating that no synthesis of ACh occurred. In the absence of HC-3 high-[K^+] medium caused a similar decrease of the ACh content of the tissue, but much more ACh was released into the medium than disappeared from the tissue, showing that synthesis of ACh took place, stimulated under the influence of high [K^+].

In summary it is unlikely that the high-[K^+]-induced release of surplus ACh derived from the muscle fibres, since no surplus ACh was released as a result of their transection, by which they were depolarized selectively.

Uptake of [^3H]ACh

If surplus ACh accumulates as a result of the uptake of released ACh into Schwann cells or nerve terminals, it is to be expected that exogenous ACh is also taken up into the tissue of a hemidiaphragm whose cholinesterase is inactivated. We examined the uptake of ACh by incubating hemidiaphragms for 120 min in 5 ml medium containing 0·1 μM-[^{3}H]ACh, a concentration somewhat higher than the endogenous ACh concentration likely to arise in the synaptic cleft under resting conditions after cholinesterase inhibition (Katz & Miledi, 1977, 1981). The results presented in Table 4 show

that, after the incubation, about 4 pmol [^{3}H]ACh could be washed from the preparation within 5 min. This amount probably represented [^{3}H]ACh adhering to the surface and contained in the interstitial space of the muscle (cf. Krnjević & Mitchell, 1960). The amount of [^{3}H]ACh remaining in the muscle after washing was 2–3 pmol, that is about two orders of magnitude less than the amounts of unlabelled surplus ACh which would have accumulated in the end-plate region during the same

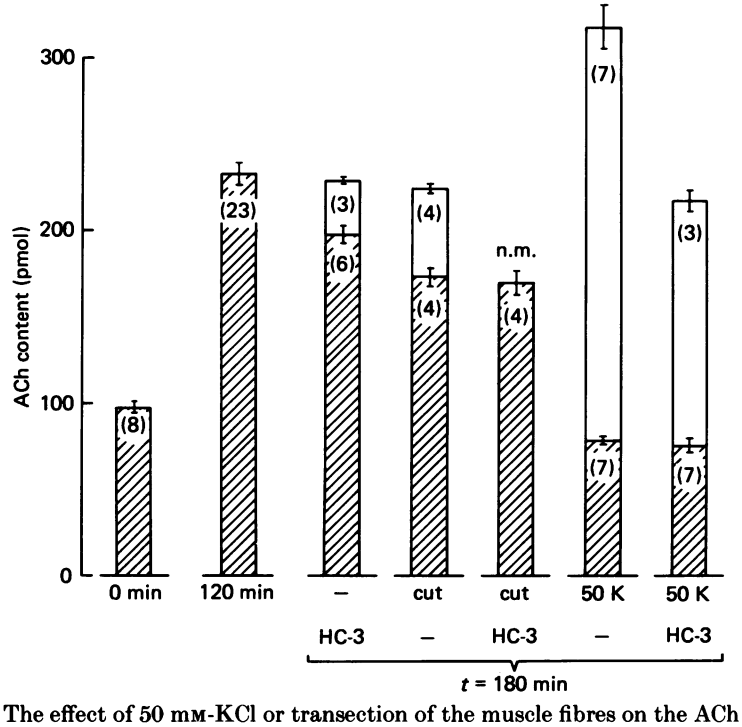

Fig. 7. The effect of 50 mM-KCl or transection of the muscle fibres on the ACh content of the rat hemidiaphragm. Hemidiaphragms were incubated for 0, 120 or 180 min at 30 °C and then extracted for determination of ACh content. Between $t = 0$ and 15 min the medium contained 0·2 μM-soman, and between $t = 120$ and 180 min 10 μM-HC-3 and/or 50 mM-KCl (50 K) in other experiments. In some experiments the muscles were cut transversely (cut) on both sides of the row of end-plates at $t = 120$ min, before being incubated during a final 60 min period. On top of the hatched columns representing extractable ACh, the amounts of ACh diffusing into the medium are presented as open columns. In the incubation period between $t = 60$ and 120 min, $29 \pm 1{\cdot}1$ (16) pmol ACh diffused into the medium ('resting release'). Means ±S.E. of mean with numbers of observations in parentheses. n.m., not measured.

period under the influence of soman. It is clear that there was no tendency of [^{3}H]ACh accumulation: [^{3}H]ACh penetrated poorly into the muscle and the final concentration of [^{3}H]ACh in the tissue after washing was about 30 pmol/g whereas that of the medium was 100 pmol/ml. Similar results were obtained in another experiment according to the same schedule. These results resemble those by Adamič (1970) who studied the uptake of 5 μM-labelled ACh after pre-treatment with tetraethylpyrophosphate as cholinesterase inhibitor: the ratio of concentration of ACh between tissue and medium was less than 1.

In conclusion, the absence of uptake of ACh by the tissue argues against the idea that surplus ACh accumulates as a result of re-uptake of released ACh.

'Bound' and 'total' ACh

As shown in Table 5 the amounts of 'bound' ACh prepared from hemidiaphragms in which surplus ACh had or had not accumulated were similar. Since the 'bound' ACh of skeletal muscle contains ACh protected against the action of added electric

TABLE 4. Uptake of [^{3}H]ACh by rat hemidiaphragm

	[^{3}H]Ach	
	(pmol)	(pmol/ml medium)
Recovered in medium after 60 min	490;527	98;105
Recovered in medium after 120 min	567;592	113;118
Recovered in washing medium	3·0;4·4	0·6;0·9
Extracted from muscle at 125 min	2·0;3·0	28;39 (pmol/g muscle)

Two soman-treated muscles (left 71·5 mg, right 77 mg) were incubated for 120 min in 5 ml Ringer medium containing 10^{-7} M-[^{3}H]ACh. The medium was renewed after 60 min. At 120 min the muscles were incubated (washed) for 5 min in 5 ml medium without added [^{3}H]ACh. After removal of the 'washing medium' the muscles were extracted for [^{3}H]ACh. During the incubation about 0·5 % of the added [^{3}H]ACh was hydrolysed, due to non-enzymic hydrolysis.

TABLE 5. 'Total' and 'bound' ACh in the rat hemidiaphragm

	'Total' ACh (pmol)	'Bound' ACh (pmol)
Without soman	123 ± 3·2 (6)	30 ± 2·0 (5)
Soman	234 ± 6·7 (6)	35 ± 1·5 (6)

The muscles were incubated for 120 min in Ringer medium containing 0·2 μM-soman in part of the experiments, and subsequently for 30 min in Ca^{2+}-free medium containing 0·5 mM-EGTA and 2 mM-$MgCl_2$. The preparations were then either extracted for determination of 'total' ACh or homogenized in Ca^{2+}-free medium containing electric eel acetylcholinesterase (5 μg/ml) at 0 °C. The homogenate was incubated for 4·5 min at 0 °C and then extracted for determination of 'bound' ACh (see Methods). Mean values ± S.E. of mean with numbers of observations in parentheses.

eel acetylcholinesterase, probably because it is occluded in synaptic vesicles (Miledi *et al.* 1982), this observation may suggest that surplus ACh is not stored in synaptic vesicles.

DISCUSSION

Origin of the resting release of ACh: possible connexion with surplus ACh

We investigated the possibility that the resting release of ACh originated from a compartment (the surplus ACh compartment) which becomes filled with ACh after cholinesterase inhibition, but remains empty when cholinesterase is fully active. This would imply that no resting release of ACh occurs without cholinesterase inhibition, because ACh synthesized in this compartment would be broken down before it could be released. Indeed, estimations of resting release of ACh gave significantly higher values in muscles treated with an anticholinesterase than in untreated muscles.

However, the release was estimated by the decrease of the ACh content of the tissue which took place spontaneously during incubation in the presence of HC-3, and it is conceivable that HC-3 did not fully inhibit the synthesis of ACh under resting conditions, so that the release was underestimated.

The observation that the resting release of ACh did not increase while surplus ACh accumulated, at first sight seemed to suggest that the resting release could not originate from the compartment in which surplus ACh accumulated. However, this inference would be valid only if the resting release of ACh occurred by electrochemical diffusion through pores in the membrane, and this may not be the case (see Katz & Miledi, 1981).

The possibility that the resting release of ACh was due to spontaneous nervous activity resulting from cholinesterase inhibition (Masland & Wigton, 1940), can be dismissed because the resting release is not reduced by tetrodotoxin which abolishes such activity (Miledi, Molenaar & Polak, 1978). It is also unlikely that soman caused ACh leakage by an action different from its anticholinesterase action, since the resting release of ACh was also observed with other cholinesterase inhibitors such as neostigmine.

The denervation experiments clearly demonstrate that resting ACh release derives mainly from a compartment located within the motor nerve endings, because resting release, just as surplus ACh formation, was greatly reduced 16–18 h after section of the phrenic nerve when the motor nerve endings had degenerated selectively. The reduction of resting release of ACh was about the same as that found earlier in 6-day-old denervated diaphragms, after degeneration of the nerve branches (Miledi, Molenaar, Polak, Tas & van der Laaken, 1982). Because the resting ACh release is mainly non-quantal (see Introduction) it probably originates from the cytoplasm of the nerve endings. The present results refute earlier suggestions that the myelinated nerve branches in skeletal muscle are responsible for the spontaneous release, notably by Evans & Saunders (1974) who found ACh synthesis and 'release' in ventral roots of the cat.

We conclude that, although the exact connexion between resting ACh release and the synthesis of surplus ACh remains to be clarified, both phenomena reflect a considerable resting synthesis of ACh in the nerve terminals (cf. Birks & MacIntosh, 1961).

Depot ACh

About 80 % of the ACh present in the rat diaphragm immediately after its isolation, is localized in the motor nerve endings since this fraction is lost after their degeneration (Miledi *et al.* 1982; present experiments). A similar proportion is available for release by prolonged nerve stimulation or incubation in a high-$[K^+]$ medium, as revealed by the stimulation-induced loss of ACh from hemidiaphragms not exposed to an anticholinesterase, in which ACh synthesis was inhibited by HC-3. The depletable ACh fraction apparently represents nearly all ACh contained in the presynaptic nerve endings. Birks & MacIntosh (1961) and Gorio *et al.* (1978) came to a similar conclusion in experiments on the cat superior cervical ganglion and the mouse diaphragm, respectively.

The amount of ACh which could *not* be released by prolonged stimulation from

hemidiaphragms with active cholinesterase in the presence of HC-3, was about equal to and probably identical with the ACh present in the denervated hemidiaphragm. The latter fraction is localized mainly in the muscle fibres where it is synthesized by an enzyme other than choline acetyltransferase, possibly carnitine acetyltransferase (Miledi *et al.* 1982) which is able to catalyse the acetylation of choline (White & Wu, 1973; Roskoski, Mayer & Schmid, 1974; Tuček, 1982).

Synthesis and hydrolysis of surplus ACh

Two aspects of surplus ACh seem very puzzling: its high rate of synthesis and its continuous hydrolysis in the absence of cholinesterase inhibition. Concerning the first point it is difficult to understand from a teleological point of view why there should be such a waste of energy in a resting muscle in the form of futile synthesis of ACh. In this connexion it should be mentioned that the initial rate of surplus ACh formation, about 3 pmol/min, was almost as high as the ACh synthesis induced by electrical stimulation of the nerve. This problem would be solved if released ACh triggers its own synthesis through an action on presynaptic ACh receptors. In that case cholinesterase inhibition might switch on the synthesis by preserving the released ACh. However, it is unlikely that either nicotinic or muscarinic receptors are involved in surplus ACh formation, since α-bungarotoxin and atropine did not prevent the process.

Concerning the second point, hydrolysis by cholinesterase of the surplus ACh immediately after its formation, it may be observed that cholinesterase is not supposed to hydrolyse ACh in its stores, but rather to attack the transmitter after its release. This paradox would be partly explained if there is re-uptake of released ACh into nervous or non-nervous elements in the muscle. In that case cholinesterase inhibition would enable the released ACh to be preserved and to be taken up into the cells (in fact such an uptake process has been found in brain tissue, Polak, 1969). However, there are two strong arguments against this idea: first, there was no accumulation into the preparation of exogenously applied [^{3}H]ACh, and, secondly, resting ACh release remained constant when the store of surplus ACh became saturated. If re-uptake had occurred, saturation of the surplus store should have led eventually to a decreased uptake and increased overflow of ACh.

In conclusion it would appear that there is intracellular cholinesterase in a compartment of the motor nerve ending in which ACh synthesis takes place. Salpeter (1967) studied the localization of cholinesterase at the motor end-plate by means of radioautography of bound radioactive diisopropylfluorophosphonate and concluded that little or no (less than 10 %) cholinesterase was located inside the nerve terminals. However, she noted that it was difficult to exclude the presence of cytoplasmic cholinesterase because of the spread of the intense staining at the synaptic cleft. Evans & Saunders (1974) found that the ACh content of the ventral roots of the cat increased upon incubation with physostigmine. Apparently, myelinated nerves do contain cholinesterase to which cytoplasmic ACh has access. Feng, Rogers & Salpeter (1974) found evidence suggesting cholinesterase activity in Schwann cells at the motor end-plate.

The anticholinesterase-induced accumulation of surplus ACh in the motor nerve ending could imply that the motor nerve ending contains a choline pool (possibly

derived from phospholipids) which normally, when cholinesterase is active, is acetylated and de-acetylated continuously by choline acetyltransferase and cholinesterase, respectively, and which is converted into surplus ACh after cholinesterase inhibition. If this idea is correct, the synthesis of surplus ACh, unlike that of depot ACh, should not be dependent on the uptake of choline from the interstitial space. Indeed it was found that [^{3}H]choline, added to the incubation medium, was incorporated into depot ACh but not into surplus ACh. The observation that HC-3 completely inhibited ACh synthesis in hemidiaphragms, in which the accumulation of surplus ACh had nearly reached its maximum, is not in disagreement with the idea, since under this condition all intracellular choline initially present, could have been used up. However, other observations indicate that the accumulation of surplus ACh is prevented by HC-3 when this compound is present in the incubation medium from the moment when the cholinesterase is inactivated (R. L. Polak, unpublished experiments). This seems to be contradictory to the idea that surplus ACh is synthesized from an intraneuronal choline pool, unless one assumes that HC-3 not only inhibits the transport of choline through the cell membrane, but also its intracellular transport to the site where surplus ACh is synthesized.

Localization of surplus ACh

The present results indicate that surplus ACh accumulates in several compartments, one of which is in the muscle fibre. However, this compartment contained only little surplus ACh, since the surplus ACh accumulating in denervated muscles was 20% of that accumulating in innervated control muscles. It is unlikely that the surplus ACh in denervated muscles was localized in endings of branches of intercostal nerves, because it accumulated in both end-plate-free and end-plate-containing regions at some distance from the costal margin.

The greater part of the surplus ACh was synthesized in the nerve terminals, since the accumulation of surplus ACh was decreased by about 80% as soon as 16–18 h after section of the phrenic nerve. This does not necessarily imply that the synthesized surplus ACh was remaining *localized* in the nerve terminals because it is conceivable that ACh diffused backwards into a pre-terminal region of the motor nerve. Indeed, if this pre-terminal region contains cholinesterase intracellularly it is possible that the surplus ACh was mainly localized in this region, and that normally, in the absence of cholinesterase inhibition, retrograde diffusion of ACh also is taking place, but limited to a very small distance because of the breakdown of ACh.

The finding that the 'bound' ACh fraction which probably is derived mainly from ACh in synaptic vesicles (Miledi *et al.* 1982), was not increased after accumulation of surplus ACh, suggests that surplus ACh was contained in a cytoplasmic compartment, and not in the synaptic vesicles.

Release of surplus ACh

In the cat autonomic ganglion, surplus ACh is unavailable for release by nervous stimulation (Birks & MacIntosh, 1961; Collier & Katz, 1971). The present results suggest that this is also true for the surplus ACh in the rat diaphragm. On the other hand, a considerable part of the surplus ACh was liberated from the diaphragm by incubation in a high-[K^+] medium, in agreement with observations in the cat ganglion (Collier & Katz, 1971).

The release of surplus ACh by high-[K^+] was dependent on the presence of Ca^{2+} in the medium, suggesting that the surplus ACh was released in the same way as depot ACh, i.e. in the form of ACh quanta. In fact, according to Vizi & Vyscočil (1979) the high-[K^+]-induced release of ACh from the anticholinesterase-treated hemidiaphragm of the mouse was mainly quantal. It is possible that surplus ACh is taken up into synaptic vesicles before being released. Perhaps the brief depolarization caused by the arrival of a nerve impulse is insufficient to bring about the release of surplus ACh in this way.

An alternative possibility is that high-[K^+] caused the release of surplus ACh in a non-quantal manner, since several, presumably non-quantal release processes have been reported to be, at least partially, Ca^{2+} dependent. For instance, the resting release of ACh from mouse and rat diaphragms is reduced in the absence of Ca^{2+} (Lang, Molenaar, Newsom-Davis & Vincent, 1984; Molenaar & Polak, 1985), and the release of ACh from synaptosomes prepared from *Torpedo* electric organ, depleted of synaptic vesicles by osmotic shock and later refilled with ACh, does not occur in the absence of Ca^{2+} (Israël, Lesbats & Manaranche, 1981). Israël, Lesbats, Manaranche & Morel (1983) have demonstrated that even ACh release from reconstituted proteoliposomes is Ca^{2+} dependent, provided that these structures are equipped with presynaptic membrane constituents. Hence it cannot be excluded (although it does not seem very likely) that surplus ACh is released in a non-quantal but Ca^{2+}-dependent fashion directly from the cytoplasm of the motor nerve ending during its continuous depolarization in a high-[K^+] medium.

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