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FREE AND BOUND ACETYLCHOLINE IN FROG MUSCLE

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SUMMARY

1. Frog sartorius muscles were divided into end-plate containing (e.p.) and end-plate-free (non-e.p.) segments or homogenized in Ringer solution at 0 °C in the presence or absence of added acetylcholinesterase from electric eel. ACh was extracted from the tissue or from the homogenates and measured by mass fragmentography.

2. The concentration of ACh in non-e.p. segments was about six times lower than that in e.p. segments.

3. Homogenization of muscles in Ringer caused the hydrolysis of a small fraction ('free-1') of total ACh; addition of extra acetylcholinesterase caused hydrolysis of another, greater, fraction ('free-2' ACh). The esterase-resistant ('bound') ACh was stable at 0 °C up to 15 min of incubation.

4. Denervation for 15 days, which caused the disappearance of the nerve terminals, did not influence ACh in non-e.p. segments, but reduced total and bound ACh by about 75 %, and free-2 ACh by 90 %.

5. Treatment with La^{3+} ions, which caused the disappearance of synaptic vesicles, did not influence total ACh, but reduced bound ACh by 75 %, whereas free-1 and free-2 ACh were increased.

6. Electrical stimulation of the nerve at 5 sec^{-1} or incubation with 50 mM-KCl did not affect ACh in the non-e.p. segments, but reduced by roughly 60 % total, bound, and free ACh.

7. It is concluded that about 75 % of bound ACh derives from synaptic vesicles, corresponding to 11,000 molecules per vesicle, and 25 % from non-neural ACh; that free-1 and free-2 ACh derive mainly from the nerve terminal cytoplasm, although they may be contaminated by vesicular ACh.

INTRODUCTION

The theory of vesicular ACh release (Del Castillo & Katz, 1957) has been challenged in recent years by results from biochemical experiments, notably those on the electric organ of *Torpedo* (reviews: Israël, Dunant & Manaranche, 1979; Tauc, 1979). For

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example Dunant, Gautron, Israël, Lesbats & Manaranche (1972), Dunant, Israël, Lesbats & Manaranche (1977) and Dunant, Corthay, Eder & Loctin (1980) found that the 'bound' ACh fraction in the *Torpedo* electroplaque, that is the ACh which during and after homogenization is protected against cholinesterase, presumably by being occluded in synaptic vesicles (Marchbanks & Israël, 1971), remained unchanged even after excessive stimulation of the nerve. On the other hand, the 'free' ACh fraction, that is the ACh which is destroyed by the esterase during homogenization and which is thought to derive from the nerve terminal cytoplasm was found by Dunant *et al.* to be depleted by nerve stimulation. Furthermore, after stimulation in the presence of radioactive precursors of ACh, both the ACh released from the tissue and the free ACh were preferentially labelled with respect to the bound ACh (Dunant *et al.* 1972; Suskiw, Zimmermann & Whittaker, 1978).

On the other hand, the theory of vesicular tansmitter release is supported by the effects of black widow spider venom (Clark, Hurlbut & Mauro, 1972) and Lanthanum (Heuser & Miledi, 1971; Miledi, Molenaar & Polak, 1977*b*, 1980; von Wedel, Carlson & Kelly, 1981) on frog muscle and of the spider venom on mouse diaphragm (Gorio, Hurlbut & Ceccarelli, 1978). Both the venom and La^{3+} ions produced a heavy but transient discharge of quanta associated with release of chemically detectable ACh and disappearance of synaptic vesicles from the nerve terminals.

In the present paper we studied the effects of various methods of stimulating ACh release on free and bound ACh in skeletal muscle of the frog. We found, among other things, that stimulation of the nerve or incubation of the muscle with 50 mM-KCl reduced both free and bound ACh and that there were two types of free ACh, one fraction which was hydrolysed instantaneously during homogenization, and another fraction hydrolysed only very slowly unless acetylcholinesterase from electric eel was added. We will report in a later paper (Miledi, Molenaar & Polak, 1982) the effect of different modes of stimulation on ACh release, before and after depletion of synaptic vesicles by La^{3+} ions.

METHODS

Preparation and incubation of muscle. The experiments were made on frogs (*Rana temporaria*), from which normal and denervated (Miledi, 1960) sartorius muscles were dissected. In some experiments the muscles were divided into end-plate-containing (e.p.) and end-plate-free (non-e.p.) segments as described earlier (Miledi, Molenaar & Polak, 1977*a*). Muscles were incubated in phosphate-buffered Ringer (composition: 116 mM-NaCl; 2 mM-KCl; 1·8 mM-$CaCl_2$; 2 mM-sodium phosphate, pH 7·2) or in Ringer of modified composition as indicated in Results. In the experiments with $LaCl_3$ the Ringer was buffered with 5 mM-Tris maleate which does not precipitate in the presence of La^{3+} (Miledi *et al.* 1977*b*).

Homogenization of muscle. Freshly dissected or incubated muscles were disrupted with an Ultra-Turrax homogenizer either at 20 °C in 3 ml. acetonitrile with 2·5% (w/v) trichloroacetic acid (TCA), or at 0 °C in 3 ml. normal Ringer. The first procedure extracts all ACh from the tissue (Miledi *et al.* 1977*a*, 1980). The homogenate in Ringer was divided into two equal portions, one to which TCA (10%, w/v, final concentration) was added within 30 sec after the beginning of the homogenization, and the other to which TCA was added after 5 min incubation at 0 °C in the presence of 1 μg/ml. acetylcholinesterase (AChE) from electric eel. The AChE hydrolysed the ACh in free solution as judged by the hydrolysis of ACh-d_9 added before its addition. After the addition of TCA, ACh-d_{16} was added as an internal standard for the determination of endogenous ACh.

Estimation of ACh. The purification of ACh was essentially that of Molenaar & Polak (1976), which is a modification of the procedure by Welsch, Schmidt & Dettbarn. (1972). After deproteination

of the sample by centrifugation and repeated precipitations of ACh by KI_3 in the presence of tetramethylammonium bromide as a carrier (Polak & Molenaar, 1974) the ACh was analysed on a gas chromatograph/mass spectrometer (Jenden, Roch & Booth, 1973; Polak & Molenaar, 1974, 1979). Samples of ACh in homogenates in Ringer were purified as described earlier for the measurement of ACh in incubation media (Miledi *et al.* 1980). Endogenous ACh, ACh-d_9, and ACh-d_{16} were monitored simultaneously at m/e 58, 64 and 66, respectively .

TABLE 1. Effects of denervation and stimulation on junctional and extrajunctional ACh in frog muscle

		n	Weight (mg)	ACh content (p-mole)	ACh concentration (p-mole g^{-1})
Normal muscle	e.p.	21	42±2·1	36±2·8	900±67
	non-e.p.	21	8·3±0·5	0·9±0·2	130±32
Muscle 15 days after denervation	e.p.	6	42±2·1	6·4±0·6	160±35
	non-e.p.	6	8·5±0·6	1·4±0·2	160±7
Muscle stimulated 60 min at 5 sec^{-1} or 50 mM-KCl	e.p.	9	44±3·8	13±1·4	310±39
	non-e.p.	7	9·5±1·0	1·3±0·3	160±33

Muscles were divided into segments containing end-plates (e.p.) and segments without end-plates (non-e.p.), which were subsequently extracted for estimation of ACh. Before the division, some muscles were stimulated during 60 min either by electrical stimulation of the sciatic nerve (5 sec^{-1}) or by KCl (50 mM). The results from the stimulation experiments were pooled. Means±S.E. of the mean.

Materials. AChE and tetrodotoxin (TTX) were from Boehringer Mannheim and ACh-d_{16} from Merck, Sharp & Dohme. ACh-d_9 was synthesized earlier (Polak & Molenaar, 1974). Diethyldimethylpyrophosphonate (DEPP) was kindly provided by Dr H. P. Benschop, Prins Maurits Laboratory, Institute for Chemical and Technological Research, TNO, Rijswijk, The Netherlands.

RESULTS

Neural and non-neural ACh

Frog sartorius muscle contained about 40 p-mole ACh that was localized mainly in the end-plate region (e.p.) of the muscle (Table 1). Denervation caused a reduction of the ACh content of the e.p. region. Its concentration, expressed in p-mole per gram of tissue, decreased to that in the end-plate-free (non-e.p.) segments. This confirms earlier observations (Miledi *et al.* 1977*a*) that part of the ACh was non-neural. However, its concentration (160 p-mole g^{-1}) was lower than that found previously (280 p-mole g^{-1}).

Incubation in Ringer medium with 50 mM-KCl, or electrical stimulation of the nerve, caused a great reduction of the ACh concentration in the e.p. segments. Apparently, ACh synthesis could not cope with release (Table 1). Stimulation did not affect the ACh concentration in the non-e.p. segments, indicating that it did not influence the level of non-neural ACh. This is in agreement with earlier findings (Miledi, Molenaar & Polak, 1981) that KCl failed to evoke ACh release from denervated muscle.

Bound ACh

The method of measuring bound ACh by homogenization of the tissue in a physiological saline solution (Dunant *et al.* 1972), can be applied to frog muscle,

provided that excess AChE is added to ensure rapid hydrolysis of free ACh. This is illustrated in Fig. 1. The hydrolysis of ACh and of added ACh-d_9 in the homogenate was slow under the influence of the esterase of the muscle; in the presence of excess AChE from electric eel all the added ACh-d_9, but only about 50% of the endogenous ACh of the muscle, was rapidly hydrolysed. The surviving ACh did not further decrease with time and, consequently, may be considered to be analogous to the AChE-resistant 'bound' ACh in the *Torpedo* electric organ.

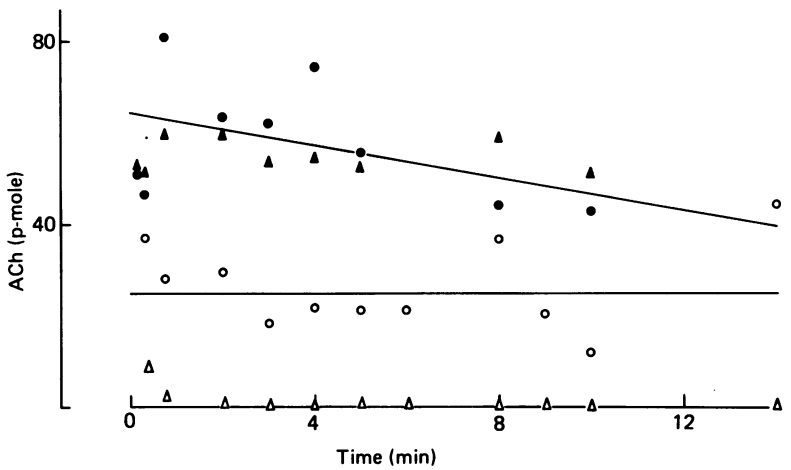

Fig. 1. AChE-resistant (bound) ACh in homogenates of frog sartorius. Homogenates were made in Ringer in the presence (open symbols) or absence (filled symbols) of AChE 1 μg/ml. from electric eel. Before homogenization 60 p-mole ACh-d_9 was added in order to follow the action of both endogenous and added cholinesterase activity. Temperature of homogenization and incubation was between 0 and 5 °C. At different times samples were taken to which TCA (10% w/v) was added for extraction of ACh, and 50 p-mole ACh-d_{16} as internal standard. Ordinate: amounts of ACh (circles, regression lines) and ACh-d_9 (triangles), in p-mole per muscle. Abcissa: time after homogenization. The values are means of two experiments.

The amount of bound ACh in the electric organ has been reported to be markedly increased after pre-incubation and homogenization in Ca^{2+}-free medium (Babel-Guérin & Dunant, 1972; Heuser & Lennon, 1973; Babel-Guérin, 1974). This may be due to decreased release of vesicular ACh during disruption of the tissue (Heuser & Lennon, 1973) and perhaps also to increased formation of synaptosomes, in which the ACh would be protected against AChE (Israël & Dunant, 1974). This phenomenon was not observed in the frog sartorius. Fig. 2 shows that the same amount of bound ACh was found (24 p-mole) regardless of whether incubation and homogenization were done in normal Ringer medium or in Ca^{2+}-free medium that contained in addition 4 mM-$MgCl_2$ (and 1·6 μM-TTX to suppress conduction of nervous impulses possibly generated by disruption of the tissue).

If bound ACh represents the ACh occluded in synaptic vesicles, it should vanish in muscles incubated with lanthanum ions, as a result of which the nerve terminals lose their vesicles, but still contain ACh (Miledi *et al.* 1980). Fig. 2 shows that bound ACh was indeed strongly reduced, though not abolished after La^{3+} treatment.

Free ACh

The fraction of free ACh, viz. the ACh that is accessible to AChE on homogenization, cannot be measured directly, but it is calculated as the difference of two direct measurements, that of total and that of bound ACh. The free ACh calculated from the data in Fig. 1 was roughly 50 % of total ACh, assuming that the ACh extracted within 30 sec from the homogenate in Ringer medium, without added AChE, gave

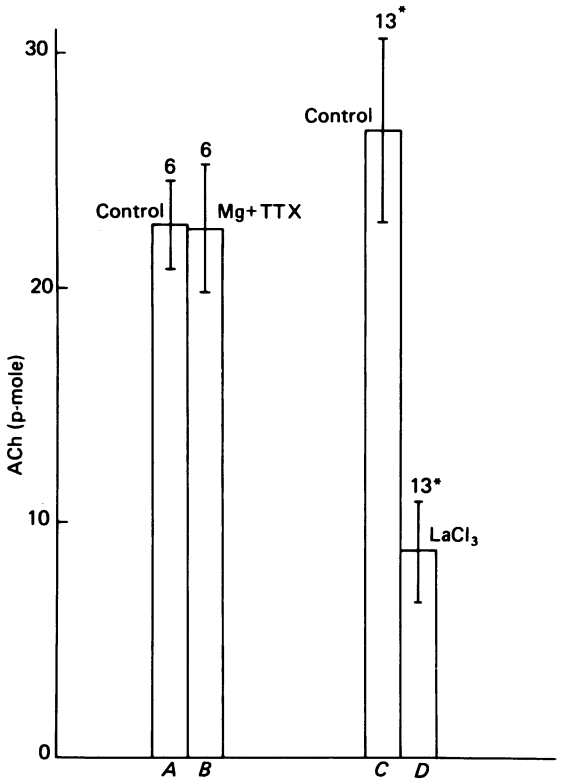

Fig. 2. Influence of Ca^{2+} and La^{3+} ions on bound ACh in frog sartorius. Muscles were incubated for 30 min and thereafter homogenized in zero Ca^{2+}, 4 mM-Mg^{2+}, 1·6 μM-TTX containing medium (*A*) or in normal Ringer (*B*, contralateral muscles). In other experiments muscles were homogenized after incubation for 5 hr either in a Tris-buffered Ringer without (*C*) or with 2 mM-$LaCl_3$ (*D*, contralateral muscles). Note: ACh-d_9 was fully hydrolysed by the added AChE in the homogenate (not illustrated in the Figure). Values are means $\pm$S.E. of the mean with number of muscles indicated in the Figure. * *C* and *D* significantly different ($P_2 < 0{\cdot}005$, Student's *t* test).

a true value of total ACh. This assumption seemed to be warranted, since no significant loss of added ACh-d_9 was found, showing that endogenous AChE activity in the homogenate was low. However, this did not exclude the possibility that during the early phase of tissue disruption, when endogenous AChE was still concentrated in the synapses, some ACh was released and hydrolysed unnoticed, that is, without concomitant hydrolysis of ACh-d_9. If this is so, we would underestimate total ACh, and, consequently, also free ACh. To test this possibility, we studied the effect of

pre-treatment of the muscle with AChE inhibitors on the recovery of ACh from the homogenate. Table 2 shows that there was indeed a loss of ACh, since the yield from DEPP or neostigmine treated muscle was significantly higher (20%) than from untreated muscles. On the other hand, no such difference was observed when muscles were homogenized in TCA-acetonitrile, our standard mixture for extracting total ACh from muscle (Miledi *et al.* 1977*a*), indicating that TCA-acetonitrile denatured the esterase rapidly enough to prevent hydrolysis of ACh during extraction.

TABLE 2. Partial hydrolysis of ACh during homogenization of frog muscle

			Extracted ACh		
	Inhibitor	*n*	Treated (p-mole)	Untreated (p-mole)	Loss of ACh (%)
Homogenization in TCA-acetonitrile	DEPP	5	40±5·1	37±3·9	8±3·3
Homogenization in Ringer followed by TCA extraction					
(*a*) normal muscles	DEPP	7	52±4·3*	39±4·9	25±5·1
	Neostigmine	3	38±9·5	30±5·2	18±6·4
(*b*) muscles treated with $LaCl_3$	DEPP	6	89±10·0*	37±5·8	55±9·2
	Neostigmine	8	42±5·2**	26±2·2	32±8·9

Muscles were homogenized in TCA-acetonitrile; alternatively they were homogenized in normal Ringer and within 30 sec the ACh was extracted from the homogenate by the addition of TCA (10% w/v, final concentration). Before homogenization the muscles were kept in Ringer with 10 μM-DEPP for 45 min, and washed for 15 min, or in 30 μM-neostigmine for 5 min immediately before homogenization without washing. Untreated control muscles were kept for the same periods in Ringer. In the experiments with La^{3+}, this protocol was preceded by incubation for 5 hr in Tris-buffered Ringer with 2 mM-$LaCl_3$. Means ±S.E. of the mean.

* $P_2 < 0{\cdot}01$; **$P_2 < 0{\cdot}025$, different from untreated controls, paired *t* test.

If the loss of ACh from muscles on homogenization in Ringer were due to the liberation and subsequent hydrolysis of vesicular ACh (cf. Heuser & Lennon, 1973), it should not occur in muscles in which the motor nerve endings have lost their synaptic vesicles under the influence of La^{3+} ions. However, as shown in Table 2, also in lanthanum treated muscles part of the ACh was lost during homogenization in Ringer, unless the AChE was inactivated. This suggests that the disappearing ACh was largely derived from the cytoplasmic compartment in the nerve terminals.

As shown in Table 2 the ACh content of muscles 5 hr after incubation with La^{3+} ions was equal to or higher than that of control muscles, in agreement with earlier findings (Miledi *et al.* 1980; Miledi *et al.* 1982). Since La^{3+} ions caused a decrease in bound ACh (Fig. 2), it follows that the free ACh fraction was *increased* under its influence.

Effect of denervation, stimulation and La^{3+} on bound and free ACh

Because there are considerable variations in ACh of sartorius muscles between different animals (Miledi *et al.* 1977*a*), it was not useful to compare total (TCA-acetonitrile extracts) with bound ACh (Ringer extracts with added AChE), on muscles from different animals, before and after stimulation. We used the following

protocol to minimize scatter of data. Each muscle was homogenized in Ringer medium, and the sample was split into two equal portions; one was extracted immediately for total ACh and the other, after addition of AChE, was incubated for 5 min and then extracted for bound ACh. This had the advantage that total, bound, and free ACh were determined in the same muscle, although as noted above (Table

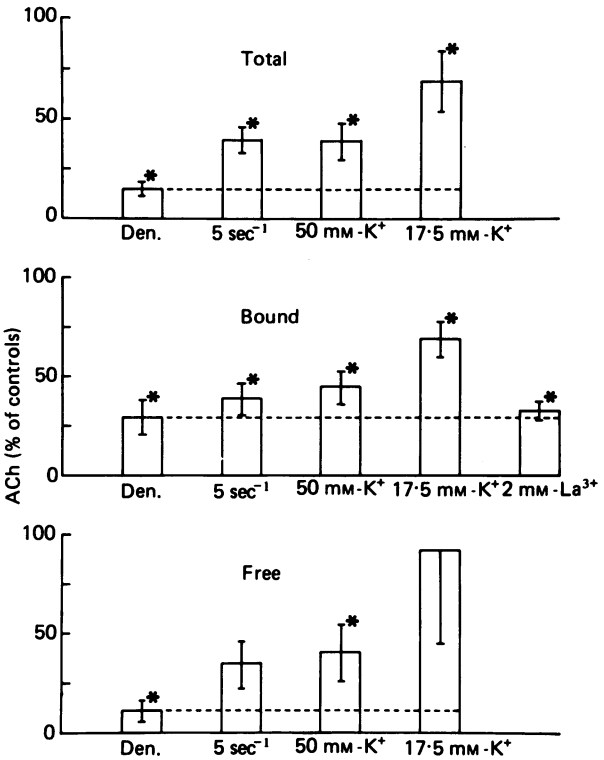

Fig. 3. Depletion of ACh in frog sartorius by various procedures. Muscles were homogenized either 14–16 days after denervation ($n = 6$), or after 60 min incubation at 20 °C under 5 sec^{-1} stimulation of the sciatic nerve ($n = 4$), or after 60 min at 20 °C in the presence of 50 mM-KCl ($n = 5$, isotonic Ringer with reduced Na^+), or after 60 min at 4 °C with 17·5 mM-KCl ($n = 5$, no correction for Na^+). For comparison the data from the experiments with 2 mM-$LaCl_3$ (Fig. 2) are also illustrated. The values are expressed as a percentage of their contralateral non-denervated or unstimulated controls. Values are means ± S.E. of the mean. * Significantly different from controls ($P_2 < 0.05$, Student's t test).

2), total and free ACh were underestimated. Contralateral muscles were used as untreated or unstimulated controls.

Fig. 3 shows that in denervated muscles total ACh had decreased by 85 %, bound ACh by 70 %, and free ACh by 90 %. Electrical stimulation of the nerve for 60 min at 5 sec^{-1}, and also chemical stimulation for 60 min with 50 mM-KCl, reduced total ACh by about 60 % and bound ACh to a level approaching that of denervated muscles; the free ACh that remained after stimulation was higher than after denervation. Mild stimulation for 60 min by 17·5 mM-KCl, causing ACh release sustained for more than

one hour (Miledi *et al.* 1982), produced an about 30 % reduction of total and bound ACh; a possible reduction in free ACh by 17·5 mM-KCl may have been masked by the large variation in the results. The effect of La^{3+} on bound ACh was about the same as that of denervation.

DISCUSSION

Since frog skeletal muscle contains a considerable amount of non-neural ACh, it was of particular importance to ascertain to which extent the fractions of free and bound ACh represented the cytoplasmic and vesicular stores of the transmitter in the nerve terminals.

The finding that bound ACh was 70 % decreased in 14 days denervated muscle, when the nerve terminals had degenerated (Birks, Katz & Miledi, 1960), indicates that 70 % of bound ACh originated from the terminals. The remainder derived from the muscle fibres because two other possible stores, the myelinated nerve branches and the Schwann cells in the muscle, contain negligibly small amounts of ACh (Miledi *et al.* 1977*a*). The bound ACh that disappeared after denervation was probably contained in synaptic vesicles because treatment with La^{3+} ions reduced bound ACh as much as denervation.

Lanthanum was an important tool in the present study, because it causes the vesicles to disappear without causing other conspicuous alterations of the ultrastructure of the nerve terminals (Heuser & Miledi, 1971; Miledi *et al.* 1980). Furthermore, it has been recently demonstrated with immunofluorescence techniques that after treatment of frog muscle with La^{3+} ions the vesicular membrane becomes incorporated into the terminal membrane (von Wedel *et al.* 1981). The nerve terminals retain the capacity to synthesize and store ACh in their cytoplasm (Miledi *et al.* 1980), as witnessed in the present experiments by the high total extractable ACh in muscles 5 hr after incubation with La^{3+}.The finding that in these muscles the bound ACh was reduced to post-denervation levels indicates that the cytoplasmic ACh was *completely* hydrolysed and that homogenization did not lead to appreciable formation of synaptosomes, in which cytoplasmic ACh might have been protected against AChE. Therefore the bound ACh was not contaminated by cytoplasmic ACh.

As far as free ACh (the difference between total extractable and bound ACh) is concerned, it originated mainly from the nerve terminals as it was reduced by 90 % after denervation. Two free ACh fractions were distinguished: free-1 ACh which was hydrolysed during homogenization by endogenous AChE, and free-2 ACh which survived homogenization, escaped into the homogenization medium, and which was destroyed immediately upon addition of electric eel AChE.

The observation that both free-1 and free-2 ACh were increased in La^{3+} treated preparations, demonstrates that these increases took place in the nerve terminal cytoplasm, where also choline acetyltransferase is localized (Fonnum, 1966; Marchbanks & Israël, 1972). However, the origin of free ACh in *untreated* muscles is less certain, as a portion of vesicular ACh might have been lost, thus contributing to free ACh. Such a loss could arise in several ways. A first possiblity, viz. loss of ACh from vesicles suspended in the homogenate, is unlikely because once it was isolated, the bound ACh did not decrease appreciably at 0 °C in the presence of AChE. This is in

agreement with the finding that the ACh in vesicle fractions from *Torpedo* electroplaque is firmly bound and protected against AChE, at least in cold isotonic medium (Marchbanks & Israël, 1971). A second possibility is that exocytosis of synaptic vesicles occurred at the moment of partial disruption of the terminals, when massive Ca^{2+} influx might be expected. However, no increase in bound ACh was found when the tissue was homogenized in low Ca^{2+}/high Mg^{2+} medium. This might indicate that complete disruption was too fast for such an effect to be important. In contrast, it has been reported that in *Torpedo* electroplaque bound ACh was increased after pre-incubation and homogenization in the absence of Ca^{2+} (Babel-Guérin & Dunant, 1972; Heuser & Lennon, 1973; Babel-Guérin, 1974). The difference could be due to a difference in homogenization technique (electroplaque: Potter-Elvehjem less than 1000 rev./min, frog muscle: Ultra-Turrax 20000 rev./min, see small print section). Perhaps contamination of free ACh by vesicular ACh was smaller in muscle than in electroplaque. A third possibility is that a population of labile vesicles loses its ACh when the internal environment is disturbed by homogenization.

Homogenization of sartorius muscle in a glass-in-glass Potter at 250 rev./min instead of in the Ultra-Turrax, reduced the bound ACh from $27 \pm 4{\cdot}7$ to $20{\cdot}4 \pm 4{\cdot}6$ p-mole ($n = 6$, $P_2 < 0{\cdot}005$, paired t test). Furthermore, when homogenization was at 250 rev./min omission of Ca^{2+} ions and addition of 2 mM-Mg^{2+} + 10 mM-EGTA to the medium increased the bound ACh from $23 \pm 2{\cdot}6$ to $38 \pm 8{\cdot}0$ p-mole ($n = 8$, $P_2 < 0{\cdot}05$, paired t test).

The finding that stimulation caused a decrease of total extractable ACh in the frog sartorius, demonstrates that synthesis of ACh was insufficient to sustain its evoked release. This could be due to the fact that the sartorius contains exceedingly little choline acetyltransferase (Tuček, Zelená, Ge & Vyskočil, 1978; Molenaar & Polak, 1980). Consequently, it was possible to study the effect of stimulation on bound and free ACh, in the absence of a synthesis inhibitor, such as hemicholinium-3. Because stimulation did not affect non-neural ACh, the reductions of bound and free-2 ACh which were observed, were due to changes of ACh in the nerve terminals. The reduction of bound ACh was probably due to a reduced amount of vesicular ACh, and the reduction of free-2 ACh to decreased levels of cytoplasmic ACh. This result is at variance with reports by Dunant *et al.* (1972, 1977, 1980) that in *Torpedo* electroplaque the bound ACh fraction was practically unchanged after stimulation, but it agrees with the finding by Suskiw *et al.* (1978) that 120 nerve stimuli decreased the ACh content of a purified vesicle fraction.

The fact that free, as well as bound, ACh was reduced in the present experiments need not contradict the theory of vesicular ACh release, because it is possible that recycling vesicles (see for review: Zimmerman, 1979) take up ACh from the cytoplasm, leading to a reduction of the free ACh fraction. On the other hand, the present results do not exclude an alternative hypothesis (Israël & Dunant, 1979) that ACh release is brought about by an 'operator', which would recruit ACh directly from the cytoplasmic store. The reduction of bound ACh could then be explained by a replenishment of cytoplasmic ACh from the vesicular store.

In previous experiments the compartment of vesicular ACh in the frog sartorius was estimated to be 25 p-mole (Miledi *et al.* 1980). In these experiments it was assumed that the depletion of ACh caused by La^{3+}, after inhibition of synthesis by hemicholinium-3, was due to a loss of the vesicular store. Recycling of vesicles seems

to be little or non-existent after La^{3+} (von Wedel *et al.* 1981), but it is possible that in the initial stage of La^{3+}-treatment there was a little recycling of vesicles, leading to a reduction of cytoplasmic ACh, and, consequently, to an over-estimation of ACh in the vesicular store. The present finding that La^{3+} caused an 18 p-mole reduction of *bound* ACh, provides clearly a lower limit of vesicular ACh, because some ACh, as explained above, might have been lost during isolation of the bound ACh fraction, and because cytoplasmic ACh cannot have biased this estimate. Taken together, the earlier and the present data indicate that the vesicular compartment contains more than 18, but less than 25 p-mole ACh, or between 11,000 and 15,000 molecules per vesicle (assuming 10^3 end-plates in the sartorius, and 10^6 vesicles per end-plate: Heuser & Miledi, 1971). This value agrees well with our estimate of 12,000 molecules ACh per quantum (Miledi *et al.* 1982) and suggests that the whole content of a vesicle is discharged as a quantum.

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