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THE EFFECT OF LANTHANUM IONS ON ACETYLCHOLINE IN FROG MUSCLE

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SUMMARY

1. Frog sartorius muscles were treated with an irreversible cholinesterase inhibitor and then incubated in Ringer with 2 mM-$LaCl_3$. The amounts of ACh in the tissue and medium were assayed by mass fragmentography, miniature end-plate potentials (min. e.p.p.s) were recorded and the end-plate was investigated by electron microscopy.

2. Addition of La^{3+} caused in normal, but not in denervated, muscles a discharge of both min. e.p.p.s and chemically detectable ACh. After 30 min both min. e.p.p.s and ACh release decreased. Between 4 and 5 hr after the addition of La^{3+} min. e.p.p.s had practically ceased and the rate of ACh release was almost back to that in the absence of La^{3+}.

3. La^{3+} caused a 50 % reduction in the ACh content of the tissue within the first 30 min; thereafter ACh gradually increased to 110 % by 5 hr. At this time synaptic vesicles were practically absent in most terminals. The ACh was predominantly located in the end-plate regions of the muscles, before as well as after the incubation with La^{3+}. ACh in end-plate free parts of the muscles was unchanged by La^{3+}.

4. Hemicholinium-3 inhibited the synthesis of ACh in the muscles, but it had almost no influence on La^{3+}-induced ACh release.

5. From these and other results, it is concluded that the ACh released by La^{3+} originates exclusively from the nerve terminals, that most likely this ACh is released via exocytosis from synaptic vesicles, and that the synthesis of ACh following the release of ACh takes place in the nerve terminals. The results further indicate that in freshly excised muscle the greater part (80–90 %) of the ACh contained in the nerve terminals is located in the vesicles.

INTRODUCTION

Exposure of a neuromuscular junction to La^{3+} ions causes a rapid and very large increase in the frequency of miniature end-plate potentials (min. e.p.p.s). Thereafter the frequency slowly decreases and eventually the min. e.p.p.s are no longer detect-

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able (Miledi, 1966; De Bassio, Schnitzler & Parsons, 1971; Heuser & Miledi, 1971). The synaptic vesicles in the endings are concomitantly reduced in number and finally they disappear in practically all the endings (Heuser & Miledi, 1971). These findings may be taken as a necessary corollary, but not as proof, of the idea that the release of the acetylcholine (ACh) packet or quantum, by which a min. e.p.p.s is produced, derives from the discharge of the content of a single synaptic vesicle. One would expect that the disappearance of the vesicles, as a result of the treatment with La^{3+}, is accompanied by a reduction of the ACh content of the nerve-muscle preparation, and that any ACh remaining in the tissue would represent a non-vesicular store, perhaps localized in the cytoplasm of the nerve terminals where ACh synthesis is thought to take place (cf. Fonnum, 1967, 1968). Furthermore, under conditions in which the cholinesterases (ChE) are inactivated, the ACh discharged from the motor nerve terminals under the influence of La^{3+} should accumulate in the incubation medium. To test these predictions we measured the content and release of ACh in the sartorius of the frog, using mass fragmentography. Part of this work has been presented in preliminary form (Miledi, Molenaar & Polak, 1977*b*). In some respects our results are similar to those recently obtained by Gorio, Hurlbut & Ceccarelli (1978), who studied the effect of black widow spider venom on ACh in the mouse diaphragm.

METHODS

Preparation and incubation of muscle. The experiments were made on sartorius muscles of male and female frogs (*Rana temporaria*, unless otherwise stated). In some frogs, the sartorius of one leg was denervated under ether anaesthesia by cutting the sciatic in the pelvis (Miledi, 1960). The muscle of the opposite leg served as control. The muscles were dissected at room temperature (18–22 °C) and the nerve cut close to its entry into the muscle. In some experiments muscles were extracted immediately after dissection to measure their ACh content; in other experiments the muscles were extracted after an incubation period. In a few cases muscles were transected into an end-plate free pelvic segment (non-e.p.) approximately 4 mm long, a longer segment containing all the end-plates (e.p.), and a very small end-plate free tibial segment which was usually discarded (Miledi, Molenaar & Polak, 1977*a*). The non-e.p. and e.p. segments were then extracted.

Incubation was usually as follows. The muscle was pre-incubated for 60 min at room temperature in 5 ml. Ringer (composition: 116 mM-NaCl; 2 mM-KCl; 1·8 mM-$CaCl_2$) to which 0·1 mM-diethyl-dimethylpyrophosphonate (DEPP), an irreversible ChE-inhibitor, had been added. It has been found that this treatment causes about 99·5% inhibition of ChE (Miledi *et al.* 1977*b*). Subsequently the DEPP was washed from the preparation with excess Ringer for 30 min, after which it was incubated at 18–22 °C in 5 ml. Ringer buffered with 5 mM-Tris maleate (pH 7·0) with or without 2 mM-$LaCl_3$. Phosphate could not be used for buffering because it would precipitate the La^{3+}. Notwithstanding the presence of buffer, the pH of the medium gradually decreased during incubation in the presence of $LaCl_3$, although it did not fall below 6. This change in pH must have been due to a reaction between La^{3+} and substances (possibly phosphates) leaking from the tissue. Formation of precipitates occurred, especially at the pelvic side of the muscle where bits of neighbouring muscles were left attached.

At different times during incubation the medium was withdrawn for estimation of ACh. Immediately after collection the samples were acidified to pH 4 with HCl to prevent hydrolysis of ACh during storage.

Estimation of ACh. The ACh was extracted from the tissue by 2·5% trichloroacetic acid (TCA) in acetonitrile, and thereafter purified by being precipitated three times in succession with KI_3 according to the method of Polak & Molenaar (1974) and Miledi *et al.* (1977*a*), which is

a modification of that of Welsch, Schmidt & Dettbarn (1972). To isolate ACh from the incubation media the ACh was precipitated first by KI_3, and the precipitate was then deproteinated by TCA in acetonitrile. Thereafter the ACh was purified further by two precipitations with KI_3. Fully deuterated ACh (ACh-d_{16}) was used as an internal standard. It was added to the tissue extracts and the incubation media before the first precipitation with KI_3.

Since hemicholinium-3 (HC-3) was found to be precipitated with KI_3 and to interfere with mass fragmentography of ACh, it was removed from the samples before the first KI_3 precipitation. This was done by chromatography on Amberlite CG 50, 100–200 mesh, as described earlier for the separation of a ACh and physostigmine (Molenaar & Polak, 1976).

The purified ACh was pyrolysed on glass at 250 °C in the entrance of a Finnigan gas chromatograph/quadrupole mass spectrometer (type 3100–003D). For chromatography a packed capillary column (i.d. 0·5 mm) was used which was directly coupled to the mass spectrometer. The method has been described in detail elsewhere (Polak & Molenaar, 1979).

For blanks a part of the extracted tissue, or of the collected medium, was used in which the endogenous ACh had been destroyed by electric eel acetylcholinesterase (Miledi *et al.* 1977*a*) and to which the internal standard had been added after inactivation of the acetylcholinesterase by heating. Alternatively, 5 ml. Tris maleate Ringer was treated according to the same procedure as the incubation media. The blanks obtained with either procedure were similar indicating that the tissue did not contain or release any substance not identical with ACh but mimicking its mass fragmentographic properties.

Uptake of ACh. The muscles were treated for 1 hr with 100 μM-DEPP in Tris maleate buffered Ringer. Subsequently, they were incubated for 1 hr in the presence of [^{3}H-acetyl]choline (160,000 c.p.m./ml.; 1, 10 or 100 μM). Thereafter the muscles were washed for 2 h in medium which was refreshed each 15 min in order to remove radioactive material from the interstitial space of the tissue. About 98–99% of the [^{3}H]ACh, present in the tissue immediately after the incubation with the labelled ACh, was removed by the 2 hr washing period. In contrast, less than 10% of endogenous ACh was lost from the tissue by washing, as calculated from the resting release of ACh. The tissue was homogenized in 2 ml. Ringer and [^{3}H]ACh and [^{3}H]acetate were measured by liquid scintillation counting according to the methods used for the estimation of choline acetyltransferase and ChE (see below). It was established that virtually all radioactivity (90–95%) in the tissue was unchanged ACh.

Synthesis and hydrolysis of ACh by homogenates of muscle. Muscles were cut into fine pieces and subsequently homogenized by means of a glass-in-glass homogenizer in medium containing 100 mM-K phosphate and 0·1% Triton X-100 (w/v).

ACh synthesis in the homogenate was estimated by measuring the conversion of 2 mM-choline and 25 μM-[^{14}C-acetyl]CoA into [^{14}C-acetyl]choline during 10 min incubation at 20 °C in the presence of 0·2 mM-physostigmine salicylate, according to the method of Braggaar–Schaap (1979) which is a modification of Fonnum's (1975) method. This method gives very low blanks, which is a prerequisite for measuring the very low ACh synthesizing activities of frog muscle.

ChE activity was assayed by a method similar to that of Hall (1973) and Johnson & Russel (1975). [^{3}H-acetyl]choline was added to homogenate to a final volume of 0·2 ml. (10,000 c.p.m., 10 μM-ACh), and hydrolysis of ACh was allowed to proceed for 15 min. The reaction was stopped by the addition of 0·1 ml. 10 M-acetic acid. The formed [^{3}H]acetate was determined by liquid scintillation counting.

Materials. Electric eel acetylcholinesterase was obtained from Boehringer Mannheim and the radiochemicals were from Amersham. ACh-d_{16} was from Merck, Sharp and Dohme and $LaCl_3.7H_2O$ from UCB. Diisopropylfluorophosphonate (DFP) and DEPP were kindly given to us by Dr H. P. Benschop, Chemical Laboratory/RVO-TNO, Rijswijk-Z.H., The Netherlands. Hemicholinium-3 (HC-3) was from Aldrich. In preliminary experiments it was found that HC-3 contained an impurity which interfered with the mass fragmentography of ACh. Since this impurity could not be removed by chromatography on Amberlite CG 50, the HC-3 was purified by recrystallization from ethanol. This completely removed the impurity as was shown by mass fragmentography.

Electron microscopy. Muscles were fixed at room temperature for about 4 hr in 2% OsO_4 in veronal acetate buffer and dehydrated with ethanol. Small specimens taken from the end-plate regions were embedded in Araldite and thin sections were stained with uranyl acetate and lead.

RESULTS

The muscles were incubated in Ringer buffered with 5 mM-Tris maleate which kept the pH between 6 and 7, and which did not precipitate the lanthanum. In contrast to muscles incubated in unbuffered Ringer containing 2 mM-$LaCl_3$, as used previously to avoid precipitation of La phosphate (Heuser & Miledi, 1971), the muscles did not become stiff, opaque and inexcitable, and the muscle fibres still contracted when transected. The Tris maleate did not cause gross changes in the amplitude or frequency of min. e.p.p.s. When 2 mM-$LaCl_3$ was added to the bath fluid, the min. e.p.p. frequency was greatly increased within 1 min. During the first hour the frequency was too high to be measured directly (cf. Heuser & Miledi, 1971), but afterwards individual min. e.p.p.s became again distinguishable and their frequency gradually decreased to a few per second in subsequent hours. At this stage their amplitude was considerably smaller than normal. Between 4 and 5 hr after the addition of $LaCl_3$, min. e.p.p.s had practically ceased at most end-plates.

The electron microscopic structure of end-plates exposed to La^{3+} was as described previously (Heuser & Miledi, 1971). After 5 hr exposure to La^{3+} most end-plates appeared as illustrated in Pl. 1*A*. Mitochondria, cysterna and glycogen granules could be seen, but the terminals were practically devoid of synaptic vesicles. Other terminals still contained many tubules and cysternae (cf. Heuser & Miledi, 1971) as well as a few synaptic vesicles, but even in these terminals the number of synaptic vesicles present was only a small fraction of that seen in control end-plates (Pl. 1*B*). In the same experiment other muscles were examined electrophysiologically. At most end-plates no min. e.p.p.s were detected over the 2–4 min observation period, while at other end-plates min. e.p.p.s occurred at a frequency of only 1–2 per min instead of the few per second seen in untreated muscles.

Release of ACh

On the assumption that DFP would prevent the hydrolysis of released ACh, we used this substance in our initial experiments. When, after removal of DFP, the incubation was begun, some 3–4 p-mole ACh were found in the medium after 1 hr. Surprisingly, subsequent incubation in medium containing $LaCl_3$ did not result in a significant increase in the amount of the collected ACh (Fig. 1), notwithstanding the fact that La^{3+} caused a tremendous increase in the frequency of min. e.p.p.s. This apparent paradox was solved when we found that ChE was inhibited by only 30–40% by 1 hr incubation in 20 μM-DFP, and consequently the ACh released by La^{3+} was presumably destroyed after such treatment. Several anti-ChE compounds were tested, and DEPP was found to be an extremely effective, irreversible, inhibitor: at 1 μM it inhibits more than 95% of the enzymic activity within 15 min and at 100 μM 99·5% (Miledi *et al.* 1977*b*). Therefore, DEPP instead of DFP was used in all subsequent experiments in which the ACh was to be collected. In this case, incubating the muscle in the presence of La^{3+} increased the amount of ACh collected per hour from 2 to 30 p-mole. Following the first hour the rate of ACh release, like the min. e.p.p. frequency, rapidly decreased to a level which after 2–4 hr was not different from that found in muscles incubated in the absence of La^{3+}. This is shown in Fig. 1 which in addition demonstrates that in the hour preceding the incubation with

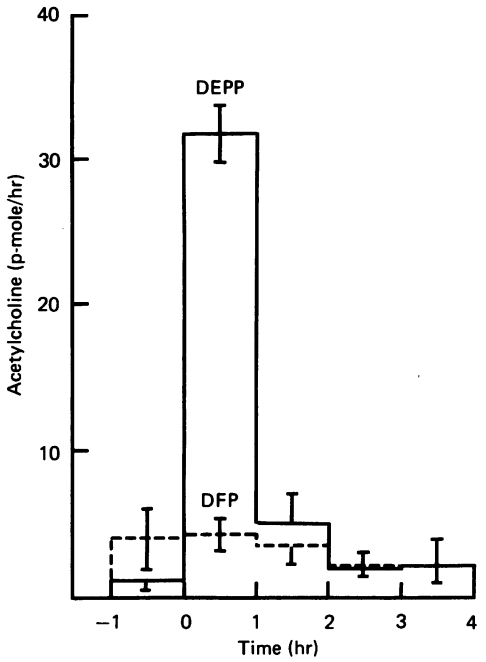

Fig. 1. Effect of $LaCl_3$ on the amounts of ACh collected during incubation of frog sartorius. The muscles were treated for 1 hr with 100 μM-DEPP (continuous line) or 20 μM-DFP (interrupted line). The medium was collected in 1 hr periods. Except for the first period, the Ringer contained 2 mM-$LaCl_3$. Means ± S.E. of mean of three experiments.

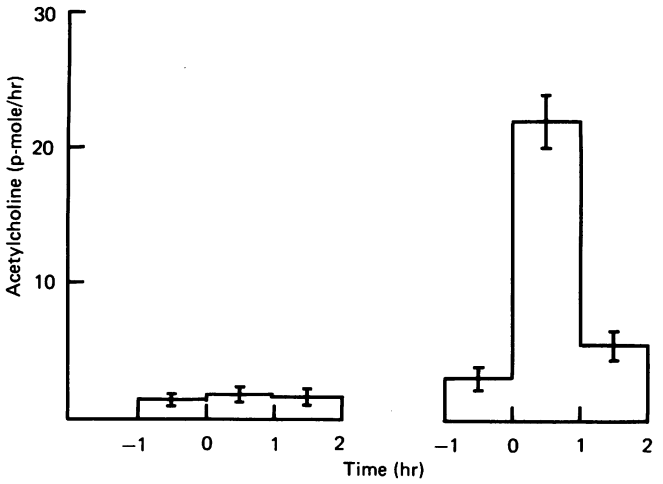

Fig. 2. Effect of denervation on the $LaCl_3$-induced release of ACh from frog sartorius. Left, denervated muscle; right, innervated contralateral control. The muscles were treated with 100 μM-DEPP. The medium was collected in 1 hr periods. Except for the first period, the Ringer contained 2 mM-$LaCl_3$. Mean ± S.E. of mean of three experiments.

La^{3+}, the amount of ACh appearing in the medium was about the same, whether the ChE was inactivated completely by DEPP or incompletely by DFP. Apparently an important part of the ACh, liberated spontaneously, escaped enzymic hydrolysis even when the ChE was incompletely inactivated. This contrasts with the liability to enzymic hydrolysis of the ACh released under the influence of La^{3+}. The identity of the ACh released, as well as that in the tissue, was ascertained by the fact that the mass-spectrometric signal was practically abolished (to about 0·2 p-mole) by treating the samples with eel acetylcholinesterase.

As reported earlier (Miledi *et al.* 1977*a*) about 25 % of the ACh in the frog sartorius is stored outside the nerves, probably in the muscle fibres. Moreover, the ACh in the extraneural store (like the neural ACh) contributes to the spontaneous release of transmitter from a sartorius preparation (Miledi *et al.* 1977*b*). It was therefore possible that the effect of La^{3+} on ACh release might be due in part to increased leakage of ACh from an extraneural store. To investigate this possibility, the release of ACh was examined in denervated muscle, where, for obvious reasons, the neural store is absent. As shown in Fig. 2, after denervation the amount of ACh released spontaneously decreased to about 50 % of control values and La^{3+} completely failed to increase the release of ACh.

ACh in the tissue

Since $LaCl_3$ causes a depletion of synaptic vesicles in motor nerve terminals, it was of interest to see whether La^{3+} causes also a decrease in the ACh content of the tissue. However, after a 5 hr period of incubation with $LaCl_3$, viz. when all vesicles had disappeared, the ACh content of the preparation was unaffected or perhaps even increased. Apparently the La^{3+}-induced loss of ACh from the tissue was replenished by the synthesis of ACh. To investigate this matter further, the release of ACh, its content in the tissue, and its rate of synthesis were analysed more precisely during the 5 hr incubation period. As shown in Fig. 3 the rate of release of ACh reached a maximum (50 p-mole/hr) as early as 15 min after the addition of $LaCl_3$; it then remained at this level for another 15 min after which it decreased sharply within the next 30 min. It was observed in other experiments (not illustrated in Fig. 3) that the time course of ACh release was the same when $LaCl_3$ was present in the first 15 min only; the action of La^{3+} is clearly irreversible. In the first 30 min, coinciding with the maximal rate of min. e.p.p.-discharge, the ACh content of the tissue decreased to about 50 % of its original level. Subsequently, the tissue ACh slowly increased until it was restored to the original level after 4 hr of incubation. It should be noted that in spite of this recovery the release of ACh was quite low after 4 hr.

The synthesis of ACh was determined from the net increase of ACh in the whole incubation system (tissue + medium). Fig. 3 shows that the synthesis, perhaps after a lag period of about 30 min, proceeded at a more or less constant rate (corresponding to about 15 p-mole/hr).

The effect of hemicholinium-3 (HC-3)

Table 1 shows that HC-3, a substance thought to block ACh synthesis in cholinergic systems by interfering with high-affinity uptake of choline (see for instance

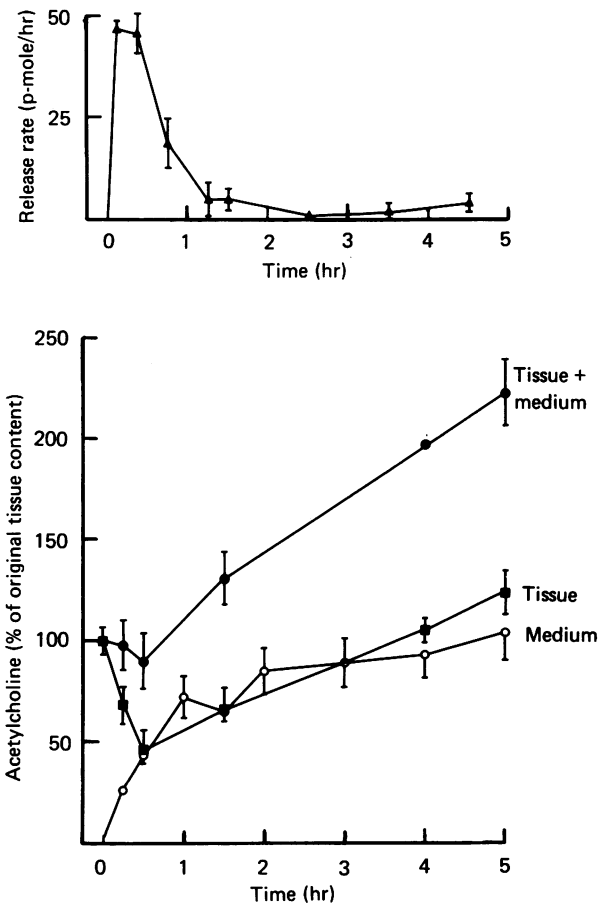

Fig. 3. Effect of $LaCl_3$ on the release, content and synthesis of ACh in frog sartorius. The muscles were preincubated for 1 hr with 100 μM-DEPP. The Ringer contained 2 mM-$LaCl_3$ from time 0; the medium was collected in periods of 15, 30 and 60 min. Upper graph: ACh release rate (p-mole/hr, ▲—▲), lower graph; cumulative ACh release (○—○), ACh extracted from the tissue (■—■), and total amount of ACh in the incubation system (tissue + medium, ●—●). The values are expressed as a percentage of the ACh content at time 0 (49 ± 3·1 p-mole), determined in the matching contralateral muscles. Mean ± S.E. of mean from determinations in at least four muscles.

Birks & MacIntosh, 1961; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973; Simon, Mittag & Kuhar, 1975), completely blocked the La^{3+}-induced synthesis of ACh in the sartorius. In the presence of HC-3, La^{3+} ions caused a marked reduction of the ACh content of the tissue. Table 1 further shows that during the first hour of incubation with La^{3+} when the rate of ACh release was highest, the rate of ACh release was not affected by HC-3, whereas the release in the whole 5 hr period seemed to be somewhat decreased.

TABLE 1. Inhibition of synthesis of ACh by hemicholinium-3

Medium	ACh released in 1 hr	ACh released in 5 hr	ACh content after 5 hr	ACh synthesis in 5 hr
Ringer	1 ± 0·4 (3)	5 ± 0·5 (2)	41 ± 5·2 (17)	−6*
Ringer + 2 mM-$LaCl_3$	32 ± 1·2 (9)	42 ± 2·5 (9)†	52 ± 5·7 (21)‡	42*
Ringer + 2 mM-$LaCl_3$ + 10 μM-HC-3	29 ± 2·2 (4)	33 ± 2·8 (6)†	17 ± 2·6 (6)‡	−1 ± 1·9 (6)**

Frog sartorius muscles were incubated for 5 hr in Tris-maleate Ringer after 1 hr preincubation with 100 μM-DEPP. When HC-3 was used, it was introduced into the medium 1 hr before the addition of $LaCl_3$. Synthesis of ACh was calculated as ACh (released) + ACh (in tissue at 5 hr) − ACh (in tissue at 0 hr). Values are expressed in p-mole ACh ± S.E. of mean with number of observations in brackets.

* The ACh content at 0 hr was assumed to be 52 p-mole.

** Mean ± S.E. of mean of synthesis values, calculated for six experiments in which, in paired controls at 0 hr, i.e. when $LaCl_3$ was added, the ACh content was 52 ± 11 p-mole.

† 33 and 42 p-mole significantly different ($P_2 < 0{\cdot}05$).

‡ 17 and 52 p-mole significantly different ($P_2 < 0{\cdot}001$).

TABLE 2. Localization of ACh in sartorius after incubation with $LaCl_3$

Experimental conditions	n	ACh concentration (p-mole.g^{-1}) e.p.	non-e.p.
Sartorius, 5 hr in Ringer	6	740 ± 88	250 ± 37
Sartorius, 5 hr in 2 mM-$LaCl_3$	6	900 ± 117	220 ± 33
Denervated sartorius, 5 hr in 2 mM-$LaCl_3$	3	220 ± 30	160 ± 30

DEPP-treated muscles were incubated for 5 hr at room temperature. After the incubation they were divided into end-plate-free (non-e.p.) and end-plate-containing (e.p.) segments; the segments were extracted for the estimation of ACh. Values are the mean ± S.E. of mean.

TABLE 3. Leakage of ACh induced by muscle transection

Experimental conditions	n	ACh content (p-mole/muscle) 2 mM-$LaCl_3$	control
5 hr incubation of intact muscle at 20 °C	8	44 ± 3	41 ± 2
2·5 hr incubation of intact muscle at 20 °C, followed by 15 hr incubation at 4 °C of			
(*a*) intact muscle	6	44 ± 7†	47 ± 4
(*b*) three muscle segments	9	52 ± 21	48 ± 7
(*c*) eight muscle segments	10	8 ± 2†‡	35 ± 5‡

Sartorius muscles, which had not been treated with DEPP, were incubated at room temperature for 5 hr. Other muscles were incubated for 2·5 hr at room temperature, cooled to 4 °C and then incubated for 15 hr, before they were extracted for the determination of their ACh content. Other muscles, prior to the 15 hr incubation at 4 °C, were either transected, 2–3 mm outside the borders of the end-plate regions, into three segments, or transected, at equal distances (4 mm), into eight segments. Values are the mean ± S.E. of mean.

† 44 and 8 p-mole significantly different ($P_2 < 0{\cdot}005$).

‡ 35 and 8 p-mole significantly different ($P_2 < 0{\cdot}005$).

Localization of ACh

To find out whether the ACh synthesized during incubation with La^{3+} is localized in the nerves or in the muscle fibres, we investigated the effect of incubation with $LaCl_3$ on the amount of ACh recovered in the end-plate containing and end-plate free parts of the muscle. The results are presented in Table 2. The concentrations of ACh in these two parts of the muscle, after incubation in the absence of La^{3+}, were similar to those previously observed in freshly extracted muscle (Miledi *et al.* 1977*a*). Table 2 further shows that incubation with La^{3+} did not alter this distribution. It was found previously that denervation causes a marked reduction of the ACh concentration in e.p. parts of the muscle, without affecting the ACh in non-e.p. parts; in fact the ACh concentration in both regions becomes equal (Miledi *et al.* 1977*a*). As shown in Table 2, the distribution of ACh in denervated muscles after incubation with $LaCl_3$ was similar to that in freshly extracted denervated muscles. These results when taken in conjunction with the finding that La^{3+} did not evoke ACh release from denervated muscle, show that La^{3+} did not cause the synthesis of ACh in denervated muscles. Apparently, in innervated muscles, the La^{3+}-induced synthesis of ACh took place in nervous structures, although it cannot be excluded that muscle tissue itself if it has nerve contacts, synthesized some ACh.

To investigate this possibility we examined the leakage of ACh from muscles which had been transected, a few mm away from the end-plate region, into three segments; or into eight segments (see Table 3). Muscles were incubated for 2·5 hr with $LaCl_3$, at which time the ACh content of the tissue should be about 80 % of its original value (cf. Fig. 3), and thereafter transected and kept at 4 °C in Ringer to allow any ACh contained in the muscle fibres to leak through their cut ends. In these experiments we did not collect the ACh in the medium and therefore we did not use DEPP. As shown in Table 3 the ACh content of preparations extracted immediately after a 5 hr incubation with La^{3+} at room temperature was about the same as after incubation in the absence of La^{3+}, and was not significantly different from that of preparations treated in the same way after inactivation of the ChE by DEPP (cf. Table 1). Table 3 shows further that a similar ACh content was found in muscles which had been incubated for 2·5 hr at room temperature and thereafter for 15 hr at 4 °C. When muscles, previously incubated in the presence or absence of La^{3+}, were transected just beyond the end-plate regions, and then incubated for another 15 hr at 4 °C, no reduction of the ACh content was found, in contrast to the 35 % reduction which was found in preliminary experiments (Miledi *et al.* 1977*b*). However, when the muscles were transected into eight segments, the 15 hr incubation was followed by a great reduction of the ACh content in the La^{3+}-treated muscles, while control muscles showed a slight, and perhaps insignificant, reduction in ACh content. This is, as if a great part of the ACh present in the muscle after incubation with La^{3+} is in a diffusable form, whereas the greater part of the ACh in control muscles is not.

Synthesis and hydrolysis of ACh by homogenates

We investigated the possibility that the stimulating effect of La^{3+} on ACh synthesis was due to a direct stimulation of enzymes involved in ACh formation. Table 4 shows that this was probably not the case. Some inhibition rather than a

stimulation was observed. It should be noted that there was also considerable enzymic activity in end-plate free segments (Table 4).

We also investigated the effect of $LaCl_3$ on the ChE of the muscle. As shown in Table 4, 2 mM-$LaCl_3$ inhibited the enzyme in homogenates of the muscle by 50%. The last line of Table 4 shows that the ChE activity in e.p. segments was about six times higher than in non-e.p. segments. This agrees with the previous results of Feng & Ting (1938) and Marnay & Nachmanson (1938). Table 4 further suggests that the ChE activity in the e.p. segments of *R. esculenta* was about double that found in *R. temporaria*.

TABLE 4. Effect of $LaCl_3$ on the synthesis and hydrolysis of ACh by homogenates of sartorius

	Frog	n	Enzymic activity (n-mole.g^{-1}.hr^{-1}) e.p.	non-e.p.
Synthesis of ACh				
Control	*R. temporaria*	4	7·6 ± 1·3	5·7 ± 1·8
2 mM-$LaCl_3$	*R. temporaria*	4	3·5 ± 0·9	1·4 ± 0·5
Hydrolysis of ACh				
Control	*R. temporaria*	3	1160 ± 32	not tested
2 mM-$LaCl_3$	*R. temporaria*	3	590 ± 12	not tested
Control	*R. esculenta*	3	2500 ± 32	400 ± 35

End-plate free (non-e.p.) and end-plate containing (e.p.) segments of muscles were homogenized; homogenates were incubated in the presence or absence of 2 mM-$LaCl_3$. Either the synthesis of [^{14}C]ACh from [^{14}C]acetyl-CoA or the production of [^{3}H]acetate from [^{3}H-acetyl]-choline was investigated. The medium included 0·2 mM-physostigmine when ACh synthesis was studied. Values are the mean ± S.E. of mean.

TABLE 5. Uptake of ACh by sartorius

Medium	[^{3}H]ACh in medium (μM)	[^{3}H]ACh in muscle (p-mole)	Concentration ratio (muscle/medium)
Ringer	1	0·9 ± 0·1 (2)	$1·1 \times 10^{-2}$
Ringer	10	5·0 ± 0·9 (4)	$0·6 \times 10^{-2}$
Ringer + 2 mM-$LaCl_3$	10	4·0 ± 0·2 (4)	$0·5 \times 10^{-2}$
Ringer	100	70 ± 10 (2)	$0·9 \times 10^{-2}$

Muscles, weighing about 80 mg, were incubated at room temperature for 1 hr in the presence of [^{3}H]ACh. Subsequently, loosely bound ACh was washed from the preparation during 2 hr. Values are the mean ± S.E. of mean.

Uptake of ACh

An active transport system is known to exist in mammalian brain, leading to the uptake of released ACh when hydrolysis of the transmitter is prevented by organophosphorus compounds with an anti-ChE action (Polak & Meeuws, 1966; Schuberth & Sundwall, 1967; Polak, 1969; Liang & Quastel, 1969). If in the present experiments with La^{3+}, the nerve terminals or muscle fibres were to take up ACh, soon after its release, the amounts of ACh collected in the medium could be a considerable underestimate of the actual amounts of ACh released by the nerve terminals. To test this,

we studied the uptake of [^{3}H]ACh in the sartorius after inhibition of the ChE with DEPP. As shown in Table 5, the uptake of ACh was very ineffective, and resulted in a concentration of [^{3}H]ACh in the tissue which was about one hundred times less than its concentration in the medium. Table 5 further shows that La^{3+} had no influence on the uptake of [^{3}H]ACh; it certainly did not promote the uptake.

DISCUSSION

La produces at least three effects on motor nerve terminals: blockage of neuromuscular transmission; massive transient discharge of ACh quanta; depletion of synaptic vesicles. Neuromuscular transmission is probably blocked because La^{3+} prevents the influx of Ca^{2+} into presynaptic nerve terminals, which normally follows depolarization of the membrane (Miledi, 1966; Heuser & Miledi, 1971; Miledi, 1971).

In some respects the action of La^{3+} is similar to that of the venom from the black widow spider which also increases the min.e.p.p. frequency, causes depletion of vesicles in motor nerve endings of the frog and induces the release of ACh in the mouse diaphragm together with a depletion of ACh in the tissue (Longenecker, Hurlbut, Mauro & Clark, 1970; Clark, Mauro, Longenecker & Hurlbut, 1970; Ceccarelli & Hurlbut, 1975; Gorio, Hurlbut & Ceccarelli, 1978). However, the spider venom seems to destroy internal structure more than La^{3+}.

La and transmitter release

While it is clear that ACh quanta, as monitored by electrical recording, are released from motor nerve terminals, it is not so clear that the *collected* ACh, measured by mass fragmentography, originates exclusively from the terminals. Indeed, there are other stores of ACh in frog muscle (Miledi *et al.* 1977*a*, *b*): (1) the nerve branches within the muscle, (2) the Schwann cells and (3) an extraneural ACh store, probably located in the muscle fibres. These stores might act as sites of ACh release, which, by being far from the ACh receptors, may escape detection with electrical recording. As pointed out previously (Miledi *et al.* 1977*a*), the amounts of ACh contained in stores (1) and (2) are very small compared to those contained in store (3) and in the nerve terminals. Therefore, the release from stores (1) and (2) is probably negligible compared to the release of ACh from the other two stores.

The fact that La^{3+} has no effect on ACh release from denervated muscle, seems to exclude the possibility that La^{3+} releases ACh from the extraneural store. This leaves the nerve terminal as the site from which ACh is released by La^{3+}. This interpretation is, of course, what would be expected from the massive discharge of min. e.p.p.s induced by La^{3+}, and is supported by the observation that the ACh released by La^{3+} is completely hydrolysed in the absence of a ChE inhibitor, in contrast to the ACh which is released spontaneously. It is known that ChE is highly concentrated at the neuromuscular junction (cf. Couteaux, 1963), and in the present experiments it was found that the concentration of ChE in e.p. segments was about 6 times higher than in non-e.p. segments. So it seems that most, if not all, of the ACh released by La^{3+} occurs in the vicinity of high density ChE, i.e. at the end-plates, and that the ACh released spontaneously originates mostly from sites with low density of the enzyme.

Katz & Miledi (1977) observed a very small end-plate depolarization in anti-

esterase treated muscle, and suggested that this might arise if the resting ACh release occurred in the form of non-quantal leakage from the motor nerve endings. The present results, however, indicate that only a small fraction of the spontaneous release comes from nerve endings, while the major part has its source at sites remote from the regions where ChE is concentrated.

The frequency of min. e.p.p.s rises to high values already within the first min after the addition of $LaCl_3$ (Heuser & Miledi, 1971). In the present experiments it was found that in the first 15 min of La^{3+} incubation the ACh release was maximal. Like the frequency of min. e.p.p.s the rate of ACh release may have increased almost immediately, but it was not possible to investigate ACh release at much shorter intervals because the diffusion of the ACh from deep end-plates to the surface of the tissue may take several minutes. None the less, the fact that the time course of ACh release in the presence of La^{3+} follows roughly the time course of the min. e.p.p. frequency, suggests that the increased amount of ACh collected after La^{3+} derives from ACh released in quantal form.

ACh in the tissue after La^{3+}

La^{3+} causes a rapid decrease of ACh in the tissue, followed by a 'recovery' of the ACh content. Thus, La^{3+} stimulates both release and synthesis of ACh. However, it is unlikely that the effect on synthesis is brought about by a direct effect on enzymes since La^{3+} did not stimulate the synthesis of ACh in a muscle homogenate.

It seems very likely that the ACh synthesized after La^{3+} treatment, the 'La–ACh', is localized in the cytoplasm of the motor nerve terminals: because there was no accumulation of 'La–ACh' in denervated muscles and because after incubation with La^{3+} the ACh was predominantly localized in the end-plate region of the muscle. However, it cannot be ruled out that in normal innervated muscle fibres, at the region of the end-plates, there is some ACh synthesized under the influence of La^{3+}. It should be noted that the formation of 'La–ACh' is not analogous to the formation of 'surplus ACh', induced by a ChE inhibitor, in the perfused superior cervical ganglion of the cat (Birks & MacIntosh, 1961; Collier & Katz, 1971), since the 'La–ACh' was also found when the ChE was not inhibited.

After multiple transection of the La^{3+}-treated muscles there was a loss of 80% of the ACh during a 15 hr incubation at 4 °C. This could mean that a great part, if not all, of the 'La–ACh' diffuses from the cut ends of the nerve branches, since no loss was observed when the muscles were transected in such a way that the end-plate region was left intact. On the other hand, no significant loss of ACh was observed when the nerve branches of control preparations were cut. Perhaps in the control preparations, there is little leakage of ACh from the cut ends of the intramuscular nerve branches because diffusion of ACh is prevented, due to its localization in synaptic vesicles which have comparatively low mobility. It is of interest to note that synthesis of an equivalent to 'La–ACh' has not been observed in the mouse diaphragm after treatment with black widow spider venom. (Gorio *et al.* 1978). These authors could not rule out the possibility that the spider venom caused the release of extravesicular ACh. In the present experiments the release of extravesicular ACh by La^{3+} seems to contribute little to ACh release since there was little ACh release from extravesicular stores at a time (5 hr) when the cytoplasmic stores were replenished or were even increased and vesicular stores practically totally depleted.

Vesicular and cytoplasmic ACh in the nerve terminals

The present experiments support the theory of vesicular release, at least as far as the release of ACh by La^{3+} is concerned: when ACh synthesis is blocked by HC-3, the release of quanta and the release of chemically detectable ACh are correlated with a reduction of vesicles and ACh in the tissue. From our data the sizes of the vesicular and cytoplasmic stores can be estimated on the following assumptions: (1) La^{3+} induces vesicular release of ACh, (2) each quantum is produced by the content of one vesicle, (3) La^{3+} does not induce a large molecular leakage of ACh from the cytoplasm of the terminals, (4) synaptic vesicles do not pick up ACh from the surrounding cytoplasm while their number is being reduced by La^{3+}. The vesicular store may then be estimated in two ways: from the reduction of the ACh in the tissue (*A*) and from the amounts of ACh released into the medium (*B*).

For the analysis we shall use the value for the store sizes reported earlier: 43 p-mole total ACh in a muscle, of which 27 p-mole are in the nerve terminals, 5 p-mole in the nerve branches and 11 p-mole in an extraneural store (Miledi *et al.* 1977*a*). Furthermore the values for the ACh content and release, that were obtained by incubation of tissue with $LaCl_3$ and HC-3 (Table 1) are used. Since the ACh content of fresh muscle varied between different sets of experiments, all present values have been normalized to 43 p-mole, which was the total fresh ACh content mentioned above. Consequently, the values from Table 1 have been multiplied by 43/52 since the control muscles contained 52 p-mole at the time when $LaCl_3$ was added to the contralateral muscles.

Calculation based on the effect of La^{3+} on ACh in the tissue (A)

The use of HC-3 prevented the loss of vesicular ACh from being masked by the synthesis of 'La–ACh'. If the Schwann cells store is ignored and the nerve branch store is taken to be 5 p-mole (Miledi *et al.* 1977*a*), and not to change during incubation, it follows that, at $t = 0$ hr,

$$\text{the total ACh content}_0 = v_0 + c_0 + m_0 + n_0 = 43 \text{ p-mole},$$

where v, c, m and n denote the vesicular, terminal cytoplasmic, extraneural and nerve branch stores. After 5 hr incubation in the presence of $LaCl_3$ and HC-3 the preparation contained $43/52 \times 17 = 14$ p-mole ACh. At that time the vesicles had disappeared, i.e. $v_5 = 0$ p-mole. Consequently,

$$v_5 + c_5 + m_5 + n_5 = 0 + c_5 + m_5 + 5 = 14 \text{ p-mole},$$

that is

$$c_5 + m_5 = 9 \text{ p-mole}.$$

Since the resting release of ACh continues during incubation with La^{3+} and since it does not appear to be influenced by HC-3, it is assumed to be $43/52 \times 5 = 4$ p-mole. This ACh originated from both stores c_0 and m_0 (Miledi *et al.* 1977*b*). Therefore,

$$c_0 + m_0 = c_5 + m_5 + 4 = 9 + 4 = 13 \text{ p-mole}.$$

At $t = 0\,h$, $m_0 = 11$ p-mole (Miledi *et al.* 1977*a*). Consequently,

$$c_0 = 13 - 11 = 2 \text{ p-mole}$$

and

$$v_0 = 43 - c_0 - m_0 - n_0 = 43 - 2 - 11 - 5 = 25 \text{ p-mole}.$$

Calculation based on La^{3+}-induced ACh release (B)

We may also calculate c and v from the ACh release evoked by $LaCl_3$/HC-3 in the first hour of incubation, that is a period in which practically all releasable ACh was collected in the

medium. Since a total of $43/52 \times 29 = 24$ p-mole was released in the presence and 1 p-mole in the absence of $LaCl_3$ (the latter practically all from non-vesicular sources, see Miledi (1977*b*), then the vesicular store, $v_0 = 23$ p-mole, and since $v_0 + c_0 = 27$ p-mole (Miledi *et al.* 1977*a*), we obtain

$$c_0 = 27 - 23 = 4 \text{ p-mole.}$$

It follows from calculations *A* and *B* that, of the total amount of ACh present in the nerve terminals, about 80–90 % is probably in the vesicles.

Measuring ACh in subcellular fractions from rodent brain or *Torpedo* electric organ, others have estimated that about 50 % in the electroplaques and somewhat less than 50 % in the brain is contained in vesicles (see for instance Israël & Dunant, 1974; Barker, Dowdall, Esmann & Whittaker, 1969). However, the amount of ACh in suspensions of vesicles is likely to be an underestimate of the amount of ACh in the vesicles *in situ* because during homogenization and subsequent fractionation of the tissue, some of the vesicular content might be lost. This is especially true as far as vesicles from the brain are concerned, which are freed from synaptosomes by incubation in a hyposmotic solution, since the vesicles have been reported to be osmo-sensitive (Marchbanks & Israël, 1971). In the *Torpedo* it has been observed that homogenization of the electric organ in an EDTA containing Ca^{2+}-free medium, which presumably prevents the destruction of labile vesicles, resulted in an increase of the apparent vesicular store from 45 to 70 % of the total ACh (Heuser & Lennon, 1973). Thus it could be that at cholinergic synapses of various systems the fraction contained in the vesicles is close to the value reported here.

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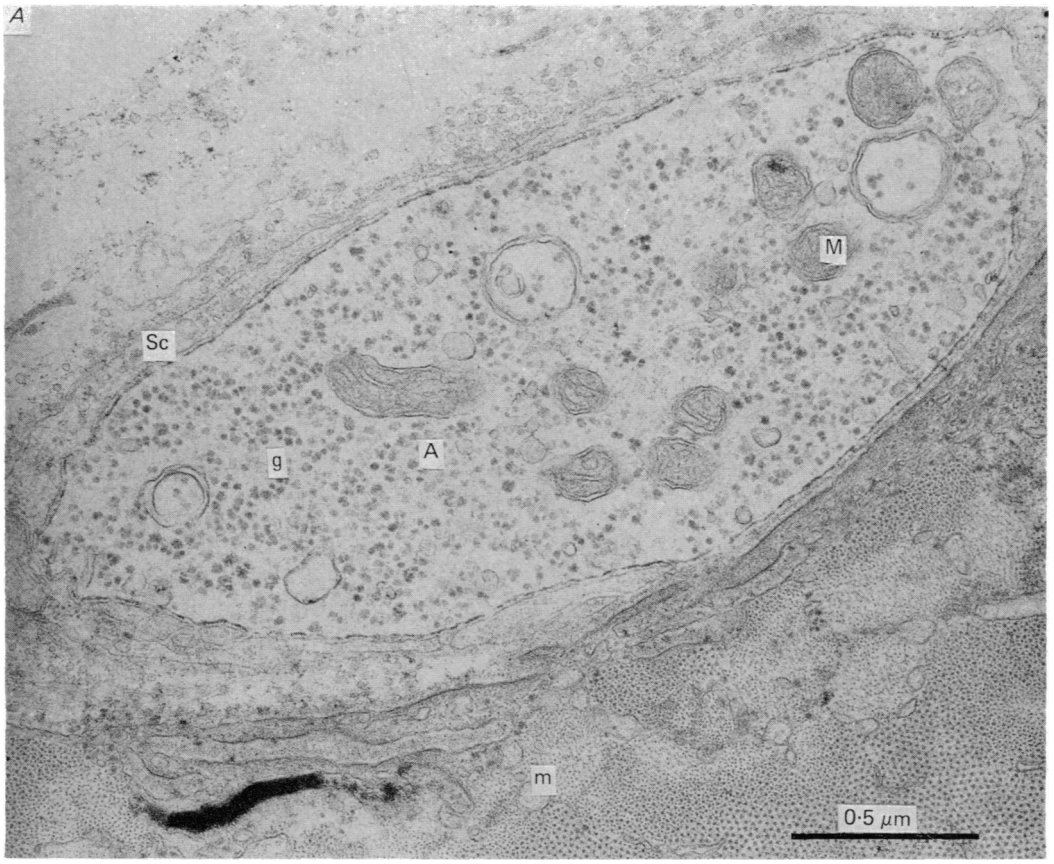
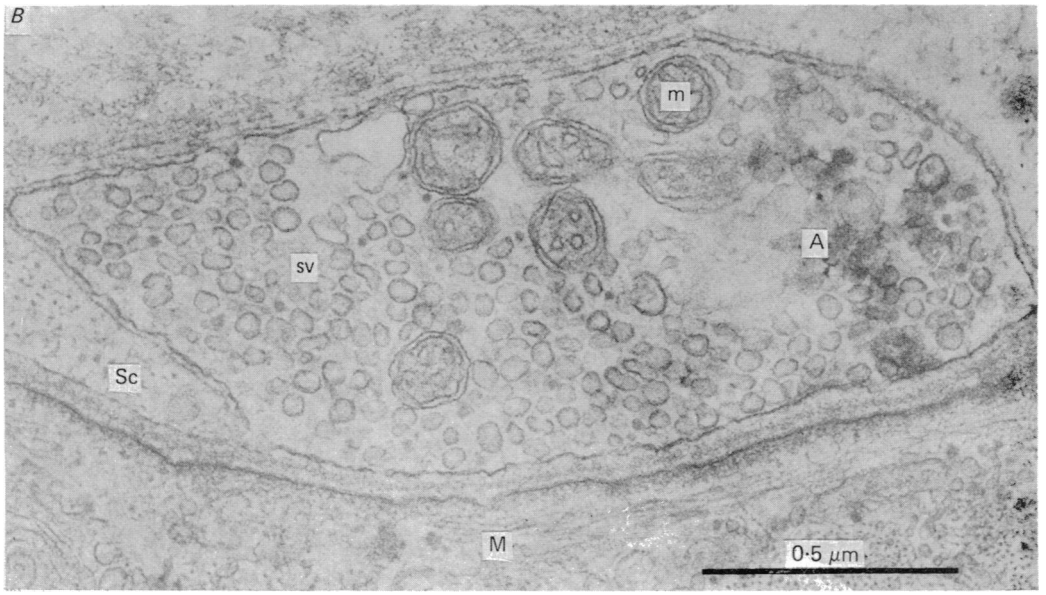
EXPLANATION OF PLATE

A, end-plate in a frog sartorius exposed for 5 hr to 2 mM-$LaCl_3$. Note the almost complete absence of synaptic vesicles. Some La^{3+} precipitate is seen on the axon membrane and, more densely, in a synaptic fold.

B, end-plate in a control sartorius muscle. Different frog from that of part *A*. The nerve terminal contains the usual amount of synaptic vesicles.

Abbreviations: A, axon; M, muscle; Sc, Schwann cell; m, mitochondria; sv, synaptic vesicles; g, glycogen.





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