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ACETYLCHOLINE CONTENT AND RELEASE IN DENERVATED OR BOTULINUM POISONED RAT SKELETAL MUSCLE

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SUMMARY

1. The acetylcholine (ACh) content and spontaneous and evoked release of ACh in rat extensor digitorum longus (EDL) muscles were determined by pyrolysis-mass fragmentography. The determinations were made on muscles paralysed by local application of botulinum toxin (BoTx) type A, on unpoisoned muscles, surgically denervated or reinnervated muscles.

2. The ACh content of unpoisoned control muscles was nearly uniform between animals and varied in the experimental series between 36 and 50 pmol. BoTx failed to affect the ACh content after 2 d of poisoning and caused a slight increase in content after 8 d. Surgical denervation reduced the ACh content within 24 h to less than 10 % of innervated muscles and upon reinnervation the ACh content was restored. Following cholinesterase inhibition the ACh content of innervated and denervated muscles increased somewhat, about equally with time.

3. Spontaneous release of ACh varied in normal innervated muscles between 40 and 100 fmol/min. In the presence of 25 mM-KCl the rate of release increased about fourfold. In BoTx poisoned muscles spontaneous release was reduced by up to 60 % of control and high potassium failed to accelerate the release at 2 d after poisoning and caused only a small increase at 8 d. Denervated muscles released ACh at a rate which was less than 20 % of control and it was not accelerated by high potassium.

4. The results show that more than 90 % of total ACh in the innervated EDL muscle is present in the nerve and its terminals. The remaining ACh is apparently formed and stored in the muscle tissue. BoTx caused a larger reduction in ACh release than can be accounted for by assuming a selective blockade of quantal release of transmitter. It suggests that BoTx has an inhibitory effect also on non-quantal ACh release.

INTRODUCTION

The purpose of the present study was to determine acetylcholine (ACh) content and release in mammalian skeletal muscle poisoned with botulinum toxin (BoTx). For comparison similar studies were made on unpoisoned, surgically denervated or reinnervated muscles. The extensor digitorum longus (EDL) muscle of young rats was

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chosen since it is of a reasonable size (40–50 mg) having about 3000 singly innervated muscle fibres (Gutmann & Zelená, 1962) and has been extensively used in electrophysiological studies on the effects of denervation-reinnervation (Sellin, Libelius, Lundquist, Tågerud & Thesleff, 1980) and of BoTx poisoning (Cull-Candy, Lundh & Thesleff, 1976). ACh determinations were made by pyrolysis-mass fragmentography as described by Polak & Molenaar (1979).

METHODS

All experiments were performed on the EDL muscle of male rats weighing 70–100 g of the Wistar strain. Surgical procedures were done using diethylether for anesthesia. Unilateral denervation was performed by removal of a 3–4 mm section of the deep peroneal nerve about 10 mm from its entrance into the muscle. For reinnervation studies, a 1 mm section of the nerve was crushed in the same location by a fine-tipped forceps. Control muscles were obtained from the contralateral unoperated side.

A powdered preparation of Cl. botulinum toxin type A (BoTx) with a mouse LD50 of about 0·05 μg/kg was dissolved in a buffer solution as described by Ambache (1949). BoTx was given in a single injection of 0·20 ml (about five mouse LD50 doses) s.c. into the anterolateral region of the right hind-leg superficial to the distal part of the tibialis anterior muscle. It produced complete paralysis of the leg within 18 h. Electrophysiological examination has shown that with this amount of toxin and mode of administration spontaneous quantal release of ACh, recorded as miniature endplate potentials, is reduced to less than 10% of normal and impulse evoked release, recorded as end-plate potentials, to less than 1% of normal (Cull-Candy *et al.* 1976). A comparison has been made between the effect on neuromuscular transmission of the BoTx toxin used in the present study with that of the isolated and purified neurotoxic component (molec. wt. about 150,000) of BoTx. In equipotent doses no difference was observed (S. Thesleff, unpublished). In the present experiments the nerve was stimulated at the time of removal of the EDL muscle, in order to confirm neuromuscular block. Control muscles were obtained from unpoisoned animals of which some had received the buffer solution.

At various times after the different procedures, the EDL muscles were excised with a peripheral nerve stump not exceeding 1 mm, under a continuous flow of oxygenated (95% O_2 plus 5% CO_2) in Krebs-Ringer solution with the following composition (mM): NaCl, 135; KCl, 5; $CaCl_2$, 4; $MgCl_2$, 1; Na_2HPO_4, 1; $NaHCO_3$, 15; dextrose, 11. The pH of the solution was 7·2–7·3.

For the determination of ACh content the muscles were weighed and homogenized in 4 ml 2% trichloroacetic acid (TCA) in acetonitrile containing 50 pmol fully deuterated ACh-d_{16} (Merck, BRD) as an internal standard. After deproteination 4 ml H_2O was added and the TCA and acetonitrile were removed by four successive washes in diethyl ether. All residual ether was removed by a stream of N_2. In order to remove choline which interferes with the ACh estimation, the ACh was precipitated 4 times in succession with potassium periodide and tetramethylammonium bromide according to the method of Polak & Molenaar (1974) which is a modification of that of Welsch, Schmidt & Dettbarn (1972). The final precipitates were dissolved in 50 μl acetonitrile and plated out on siliconized petri dishes. The acetonitrile was evaporated by heating the dishes to about 60 °C.

For estimation of ACh released the muscles were placed in oxygenated organ baths containing 5 ml Krebs-Ringer solution at room temperature, either 17–19 °C in the experiments 8 d after denervation or poisoning with BoTx or 22 ± 0·5 °C, 2 d after BoTx poisoning. Incubation was carried out for 45 min in Krebs-Ringer solution containing 10 μM-soman in order to inactivate cholinesterase, after which the preparations were washed with soman-free solution for 15 min. Thereafter incubation was continued for 2 h in Krebs-Ringer solution which was withdrawn after the first and the second hour for estimation of spontaneous ACh release. To obtain values for stimulated release of ACh the muscles were placed for 30 min in Krebs-Ringer solution containing 25 mM-potassium chloride. Its sodium chloride concentration was decreased to 115 mM for the maintenance of isotonicity. To exclude that spontaneous nerve firing might influence the results, some experiments were performed in the presence of 1·2 μM-tetrodotoxin (Sankyo, Japan). This did not influence the results. Immediately after collection of the incubation fluids, 50 pmol ACh-d_{16}

was added and they were acidified with HCl to pH 4 to prevent hydrolysis of ACh during storage. For isolation the ACh was first precipitated by potassium periodide and tetramethylammonium bromide, and the precipitates were then deproteinated by TCA in acetonitrile. The ACh was purified further by three precipitations in succession with potassium periodide. After the incubation the muscles were weighed and homogenized as described and their ACh content was determined.

Measurements of ACh were made by slow pyrolysis combined with mass fragmentography on a packed capillary column (Polak & Molenaar, 1979). The limit of determination of ACh was about 0·3 pmol.

RESULTS

The amounts of ACh extracted from innervated, denervated and BoTx poisoned muscles and the amounts of spontaneous and potassium evoked release of ACh are presented in Table 1.

TABLE 1. ACh contained in and released from EDL muscles

		ACh released (fmol/min) in				
		Normal solution		25 mM-KCl		
Procedure	ACh extracted (pmol) ($t = 0$)	60–120 min I	120–180 min II	180–210 min III	Calculated evoked release (fmol/min) IV	ACh extracted (pmol) ($t = 210$)
Controls	33±1·7 (11)	64±5·8 (11)	61±3·5 (9)	289±31 (9)	246±28 (8)	48±2·0 (10)
8 d denervated	1·5±0·34* (12)	12±5·9* (10)	7·0±2·3* (11)	26±14* (10)	19±13‡ (10)	17±1·8* (11)
Controls	33±0·8 (8)	48±6·5 (5)	39±3·3 (8)	184±24 (8)	145±23 (8)	44±2·1 (8)
8 d BoTx	44±3·5* (6)	26±2·3* (7)	28±2·7* (8)	54±7·3* (8)	26±5·9† (8)	71±2·2* (8)
Controls	52±3·1 (8)	100±6·6 (15)	93±7·3 (16)	267±16 (16)	174±18 (16)	95±4·4 (18)
2 d BoTx	54±4·9 (9)	44±5·7* (16)	37±6·3* (16)	41±8·9* (16)	3·4±7·3‡ (16)	84±2·2* (20)

The ACh content of EDL muscle was determined either after its removal from the animal ($t = 0$) or following inhibition of cholinesterase and 210 min ($t = 210$) incubation at 17–19 °C (8 d denervated and 8 d BoTx) or 22±0·5 °C (2 d BoTx). During the last 150 min of the incubation the amounts of ACh released into the medium were measured after 60 min (I), after an additional 60 min (II) and after a subsequent 30 min incubation in the presence of 25 mM-potassium chloride (III). Evoked release (IV) was calculated by subtraction of II from III. All values are means±S.E. of mean and the number of muscles used in each experiment is given by the figure within parenthesis.

* Significantly different from control (Welch's t test, $P_2 < 0{\cdot}05$).

† Significantly different from 0 (Student's t test, $P_2 = 3{\cdot}7 \times 10^{-3}$).

‡ Not significantly different from 0 (Student's t test, $P_2 > 0{\cdot}05$).

The ACh content of control muscles was nearly uniform between animals in the various experimental series, about 35 pmol, with the exception of a higher value, about 50 pmol, which was obtained 6 months later in the experiment on 2 d BoTx poisoned muscles. Following cholinesterase inhibition and 3·5 h incubation, the ACh content of the muscles increased somewhat, but to a variable degree, as seen from the last column of the Table.

Spontaneously released ACh was measured during two successive 60 min periods (columns I and II in the Table) and was found not to change with time. It varied between 40 and 100 fmol/min in different series of experiments. Evoked release of ACh was studied by incubating the muscle for an additional 30 min period in the presence of 25 mM-potassium chloride. Under this condition the rate of release of ACh increased about fourfold (column III of the Table). Since this potassium chloride concentration depolarized the nerve terminals, the release consisted of resting and depolarization evoked release. To obtain an estimate of the evoked release we subtracted the rate of resting release from the rate of ACh release in the presence of 25 mM-potassium chloride, as shown in column IV of the Table.

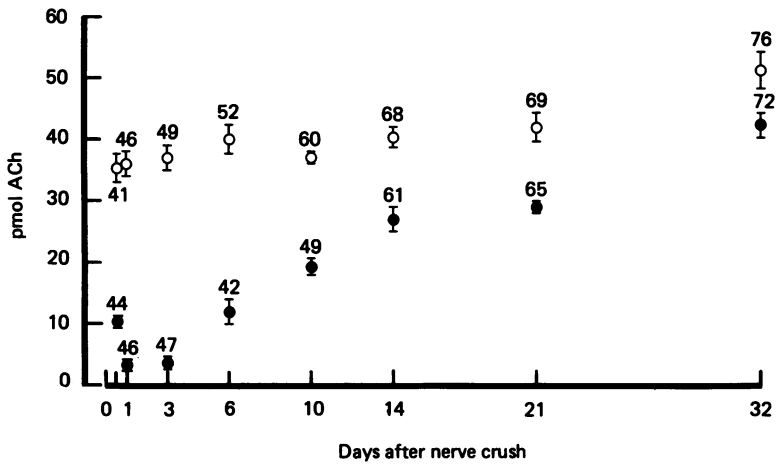

Fig. 1. ACh content (pmol) of EDL muscles at different times following denervation by crushing of the motor nerve (●) and of contralateral innervated muscles (○). The values are mean ± S.E. of mean of four to eight muscles. The figures next to each symbol indicate the mean wet weight (mg) of the muscles examined.

The ACh content of muscles which had been denervated for 8 d was only 1·5 pmol and it increased to about 17 pmol during the 3·5 h experimental period following cholinesterase inhibition. From the denervated muscles some release of ACh occurred but it was less than 20 % of that from innervated muscles, and it was unaffected by a high potassium concentration in the medium.

In contrast to denervation BoTx failed to affect the ACh content of the muscles after 2 d and even caused a significant increase in the content after 8 d (see Table 1). Spontaneous release of ACh was significantly reduced from 2 and 8 d poisoned muscles and 25 mM-potassium chloride did not cause an increase in the release 2 d after poisoning and only a small increase in muscles poisoned for 8 d.

Previous studies (Sellin *et al.* 1980) have shown that following denervation by crushing the motor nerve, reinnervation is apparent at about 9 d after the operation as shown by the reappearance of miniature end-plate potentials and a twitch in response to indirect stimulation. In Fig. 1 are shown the effects of nerve crush denervation–reinnervation on the amount of ACh extracted from EDL muscles together with respective mean wet weights of the muscles. As controls served

contralateral innervated muscles. The muscle weight increased with the age of the animal and this was accompanied by an increase in the amount of ACh extracted. Following nerve crush the mucle weight fell, compared to the control, but between 6 and 14 d it rapidly increased from 42 to 61 mg as a sign of a neurotrophic influence from reinnervation. The ACh content of denervated muscles fell by 91 % within 24 h and remained at that level for at least 3 d. However, 6 d after the nerve crush the content of ACh started to increase and between 6 and 14 d it almost doubled and reached a value of approximately 70 % of that in contralateral innervated muscles. By 32 d after the nerve crush ACh content and muscle weight approached control values.

DISCUSSION

The EDL muscle of the rat has the advantage of a uniform fibre type, being mainly a 'fast' twitch muscle. The muscle can be removed from the animal without injury and can be completely paralysed *in vivo* by local application of BoTx. Furthermore the ACh content of the muscle was found to be virtually constant between animals.

Following denervation the ACh content of the muscles dropped within 24 h by more than 90 %. During this time denervation results in cessation of transmitter release and degeneration of the nerve terminals as shown by Miledi & Slater (1970) for the rat phrenic nerve. A further indication that the nerve terminals were the main store of ACh was the increase in ACh content observed at the time of functional reinnervation.

Since nerve terminals seem to constitute the main store of ACh in the EDL muscle it is possible to estimate the average amount of ACh contained at one end-plate and in one vesicle. One muscle contains about 3000 fibres which are singly innervated (Gutmann & Zelená, 1962) and about 30 pmol neurogenic ACh (Table 1), hence about 10^{-2} pmol ACh per end-plate. According to estimates by Elmqvist & Quastel (1965) the average size of the presynaptic transmitter store in a single rat phrenic nerve terminal corresponds to 270,000 quanta. Assuming 70–80 % of the total ACh in synaptic vessels (Heuser & Lennon, 1973; Miledi, Molenaar & Polak, 1980) one gets about 17,000 molecules of ACh per vesicle.

In innervated muscles the amount of ACh increased with time when cholinesterase had been inactivated. A similar increase was observed in the denervated muscles indicating that the muscle tissue synthesized this ACh. Little is known about the mechanisms by which muscles can form ACh. However, it has been shown that enzymes able to acetylate choline are present in muscle (White & Wu, 1973; Roskoski, Mayer & Schmid, 1974; Tuček, Zelaná, Ge & Vyscočil, 1978; Molenaar & Polak, 1980). Such enzymes could account for the small amounts of ACh formed in and released from denervated muscle.

The rate of spontaneous ACh release from the rat EDL incubated at 22 °C was about 100 fmol/min, whereas that released from the rat hemidiaphragm under similar conditions was 600–1000 fmol/min (Krnjević & Mitchell, 1961; Randić & Straughan, 1964; Fletcher & Forrester, 1975; Molenaar & Polak, unpublished). Since the EDL muscle contains about 3000 end-plates (Gutmann & Zelená, 1962) and the hemidiaphragm about 10,000 end-plates (Krnjević & Miledi, 1958; Krnjević & Mitchell, 1961), the rate of ACh release per endplate in the EDL muscle was 2–3 times lower than in the hemidiaphragm.

BoTx is known to block quantal ACh release (Brooks, 1956; Thesleff, 1960). In the present study 25 mM-potassium chloride failed to evoke ACh release 2 days after poisoning and caused only a small but significant evoked release at 8 days. If it is assumed that the release of ACh evoked by high potassium is mainly of quantal nature, the small evoked release seen after 8 days of poisoning might reflect recovery of quantal release, since sprouting of motor nerve terminals has been observed at that time (Duchen, 1970, 1971).

It is believed that under resting conditions quantal release of ACh constitutes only a few per cents of total release (Mitchell & Silver, 1963; Fletcher & Forrester, 1975; Miledi *et al.* 1977). This can also be calculated from the present data assuming a miniature end-plate potential frequency of less than 2 Hz as observed in some experiments on our preparations by T. Magchielse (unpublished). Furthermore from the present observation that total ACh release was increased about 4 times by potassium chloride in a concentration reported to cause a sustained rise in the miniature end-plate potential frequency by more than 100 times (Liley, 1956), the following calculation can be made.

If quantal ACh release (A) plus non-quantal ACh release (B) constitute spontaneous ACh release (C) and if C is increased 4 times when A is raised 100 times (while B is not affected), the quantal contribution to spontaneous ACh release would amount to 3% (since $A+B=C$ and $100A+B=4C$). If potassium chloride would have raised B in addition, the quantal contribution to spontaneous release of ACh would have been still smaller.

Surprising was therefore our finding that BoTx reduced spontaneous release of ACh by up to 60%. It suggests that BoTx also has an inhibitory effect on non-quantal ACh release. This may signify that non-quantal release of ACh is a more complicated process than passive diffusion from the cytoplasm in the nerve terminal via the axolemma into the extracellular space.

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