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NEUROTROPHIC REGULATION OF MUSCLE CHOLINESTERASE: EFFECTS OF BOTULINUM TOXIN AND DENERVATION

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SUMMARY

1. In order to determine the role of acetylcholine (ACh) transmission in neurotrophic regulation of muscle cholinesterase (ChE), the effects of botulinum toxin treatment were compared with those of denervation, in terms of two fractions of muscle ChE. The sternocleidomastoid muscles of rats were either denervated or injected with botulinum toxin. After 7 days the muscles were frozen, and sequential sections were assayed for acetylcholinesterase activity. By this method, the ChE activity of end-plates (EPChE) and non-end-plate regions (background ChE, BChE) were separately determined.

2. The background ChE was reduced to the same extent by botulinum toxin and denervation. This is interpreted to mean that the neurotrophic regulation of BChE may be wholly cholinergic.

3. The EPChE was significantly reduced by both treatments, but the effect of botulinum toxin was only half as great as that of denervation. This indicates that EPChE is only partially regulated by ACh transmission. The larger effect of denervation suggests that some non-cholinergic influence may be operating in this situation as well. Disruption of the structural integrity of the end-plate, with consequent loss of binding sites for the ChE, may account for the additional effect of denervation.

INTRODUCTION

It is well known that motor nerves play an important role in the regulation of cholinesterase (ChE) activity of skeletal muscle. When the motor nerves are cut, the ChE activity decreases rapidly, although it never disappears entirely (Guth, Albers & Brown, 1964). Conversely, re-innervation of a denervated muscle restores its ChE activity (Guth & Brown, 1965).

However, the important question of how the motor nerves convey this regulatory influence to muscle has been a subject of continuing debate.

It is generally thought that one or more chemical substances released by the nerves might transmit this and other 'trophic' influences to skeletal muscles. The possibility that acetylcholine (ACh), the natural neuromuscular transmitter, might play a role in regulating muscle ChE is a plausible one, since the main function of muscle ChE is to hydrolyse ACh at the neuromuscular junction. Previous attempts have been made to test this hypothesis by blocking ACh pharmacologically, and determining the effect on muscle ChE by biochemical or histochemical methods (Stromblad, 1960; Sonesson & Thesleff, 1968). However, the results of such studies have been inconclusive, since they did not take into account the differential responses of end-plate and non-end-plate regions of muscle to neural influences (Guth, 1969). In order to resolve the problem, I have utilized a critical method by which quantitative determinations of end-plate ChE and non-end-plate (background) ChE are made separately (Guth *et al.* 1964). In the present study, the effect of blockade of ACh transmission by botulinum toxin was compared with the effect of surgical denervation. The results point not only to the important role of ACh transmission in regulation of muscle ChE, but also to a non-cholinergic effect of the motor nerve.

METHODS

Adult female rats (Charles River Strain CD) weighing approximately 200 g were used in these experiments. One group of six rats was treated by direct injection of botulinum toxin into the sternocleidomastoid (SCM) muscle. A second control group of eight rats underwent denervation of one SCM muscle, and injection of sterile Ringer solution into both the denervated and the opposite SCM muscles.

The rats were anaesthetized with I.P. chloral hydrate (0·4 g/kg), and the SCM muscles were exposed under aseptic conditions. In the first group of rats botulinum toxin was injected into the right SCM muscle through a fine no. 30 needle. Each injection of 0·03 ml. contained 1·6 ng pure crystalline type A botulinum toxin (furnished by Dr E. Schantz, Frederick, Maryland) diluted in mammalian Ringer solution immediately before use. This dose produced complete neuromuscular blockade in the SCM muscle within 24 hr, as ascertained by electrical stimulation. Because of the inevitable spread of the paralysis to the neighbouring musculature, the rats experienced some difficulty with swallowing. On the day before autopsy, each rat received 0·5 ml. of specific anti-toxin (furnished by Dr J. Ruegsegger, Lederle Laboratories, Pearl River, New York) I.P. in order to assure the safety of the laboratory personnel, but this did not alter the already established neuromuscular blockade. In the second group of rats, the nerve to the right SCM muscle was severed at its point of entry into the muscle, and excised for 0·5 cm proximally. Injections of 0·03 ml. of sterile Ringer solution were made into the right and left SCM muscles.

Since the botulinum-treated rats had difficulty in eating, owing to weakness of the pharyngeal muscles, their food was placed within easy reach in the cages, and in some instances they were hand fed. The food intake of the control rats was restricted in order to minimize the difference in nutrition between the two groups.

Seven days after the experimental procedure, the rats were again anaesthetized with chloral hydrate. The nerve to the SCM was electrically stimulated in the botulinum-treated rats, to confirm that neuromuscular blockade was still complete. The muscles were then quickly removed, placed on stiff cardboard, and immersed in liquid nitrogen. In all, six unoperated control muscles, six botulinum-treated muscles, and eight denervated muscles were assayed, as described below.

Transverse sections of the frozen muscle were cut serially, at 28 μ thickness. The first and fourth sections were placed on a slide for histochemical staining, and the second and third sections were combined in a single test tube for quantitative biochemical assay. Fourteen sections were then discarded and the sequence was repeated. By this means the muscle was surveyed histochemically and quantitatively at 0·5 mm intervals. The histological sections were fixed in 3% unbuffered glutaraldehyde and stained for AChE activity (Koelle & Friedenwald, 1949). The sections in the test tubes were assayed for AChE activity by an adaptation of Ellman's method (Ellman, Courtney, Andres & Featherstone, 1961) to a microchemical scale (Guth *et al.* 1964). After the quantitative AChE assay was performed, the sections were dissolved in NaOH and the protein content was determined by a biuret method.

The total ChE activity per muscle was determined by adding the values for all sections of the muscle, and applying the appropriate correction for the number of sections that had been discarded in the sampling procedure.

The total background ChE activity per muscle was determined by adding the activities of all the tubes for which the corresponding histological sections had no end-plates, and applying a correction factor for the sampling procedure. The BChE activity per mg protein was also computed.

The end-plate ChE (EPChE) per section was determined by measuring the ChE activity and protein content in each section containing end-plates, and subtracting the BChE, according to the following formula: EPChE/sect = Total ChE/sect − [Average BChE/mg prot × mg prot/sect].

The total EPChE per muscle was computed by adding these values for all sections that had end-plates, and correcting for the number of sections discarded in the sampling procedure.

RESULTS

The results are summarized in Table 1. Both denervation and treatment with botulinum toxin resulted in a decrease in muscle ChE. The background ChE (BChE) was reduced to the same extent in the two experimental groups. Calculated as BChE per muscle, there was a 60% loss after denervation, and a 63% loss after botulinum treatment (Fig. 1). This decrease in BChE after denervation and botulinum was highly significant ($P < 0{\cdot}001$). More important, there was no significant difference between the effects of the two treatments on BChE ($P > 0{\cdot}50$).

The end-plate ChE activity was significantly reduced in both the denervated and botulinum-treated muscles, as compared with controls ($P < 0{\cdot}01$). However, the reduction produced by denervation was nearly twice as great as that produced by botulinum toxin (59·7% cf. 31%) (see Fig. 1). The difference between the denervated and botulinum-treated groups was highly significant with respect to EPChE ($P < 0{\cdot}01$). There was clean separation of the data in the control, denervated and botulinum-treated groups of muscles (Table 2).

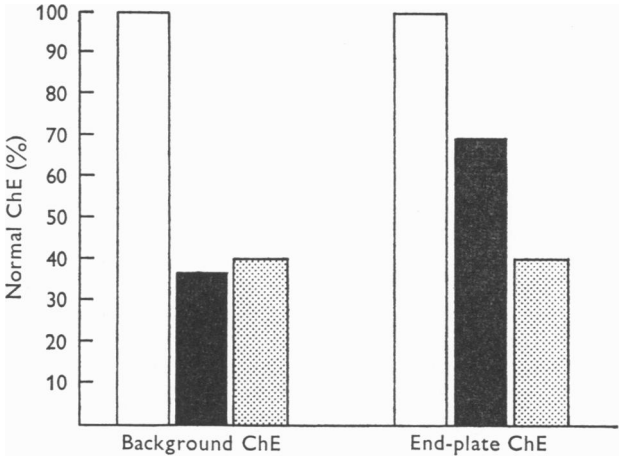

Fig. 1. Background ChE and end-plate ChE of rat sternomastoid muscle after 7 days treatment with botulinum toxin (■) or denervation (▦), expressed as percentage of control (□).

TABLE 1. Cholinesterase activity and protein content of normal, botulinum-poisoned, and denervated skeletal muscle

Experimental group	No. of muscles	Total ChE*	Total background ChE*	Total end-plate ChE*	Protein†
Unoperated control	6	13·62 ± 0·71	9·36 ± 0·61	4·25 ± 0·24	39·4 ± 1·37
Botulinum poisoned	6	6·24 ± 0·57	3·47 ± 0·40	2·94 ± 0·20	18·4 ± 2·22
Denervated	8	5·44 ± 0·39	3·72 ± 0·29	1·71 ± 0·16	19·7 ± 1·29

* μmole/whole muscle.hr ± S.E. of mean.
† Mg protein/whole muscle ± S.E. of mean.

TABLE 2. Total end-plate ChE (μmole/muscle.hr)

Control	Botulinum	Denervated
3·463	2·324	1·129
3·738	2·443	1·345
3·969	2·763	1·348
4·758	3·248	1·507
4·778	3·326	1·841
4·815	3·518	2·009
		2·097
		2·426
$n = 6$	$n = 6$	$n = 8$
$\bar{x} = 4·254 \pm 0·24$	$\bar{x} = 2·937 \pm 0·20$	$\bar{x} = 1·713 \pm 0·16$

The protein content of the botulinum-treated muscles was almost identical to that of the denervated muscles (Table 1), indicating that these treatments produced a similar degree of muscle atrophy.

DISCUSSION

There are two major findings in this study. First, the reduction of background ChE produced by botulinum treatment was equivalent to that produced by surgical denervation. Secondly, although both experimental treatments led to a reduction of end-plate ChE, the effect of denervation was significantly greater than that of botulinum toxin.

The observation that botulinum toxin and denervation both resulted in a reduction of muscle ChE is in general agreement with the two previous studies on the subject. Stromblad (1960) found a marked fall in the total ChE activity of homogenates of whole muscles, after botulinum treatment or denervation. The effect of denervation was greater than the effect of botulinum toxin in some, but not all, of his experimental groups. Since he did not distinguish between BChE and EPChE, his results cannot be compared directly with ours. Sonesson & Thesleff (1968) found that botulinum toxin and denervation both produced a reduction in the resynthesis of ChE, but the histochemical method used did not permit a valid quantitative comparison of the effects of the two treatments.

Interpretation of any pharmacological experiment depends on an understanding of the nature of the agent used. The crystalline Type A Botulinum toxin (Duff, Wright, Klerer, Moore & Bibler, 1957) used in this study is a highly potent and specific inhibitor of neural release of ACh (Burgen, Dickens & Zatman, 1949; Lamanna, 1969). Type A toxin has been reported to block both impulse-directed and spontaneous release of ACh completely in mammalian muscle (Brooks, 1956; Thesleff, 1960). It has recently been shown that Type D toxin abolishes impulse-directed ACh release completely in the frog, and reduces the frequency of miniature end-plate potentials more than 98%. The remaining m.e.p.p.s 'resemble those seen after denervation' (Harris & Miledi, 1971). The effect of the toxin appears to be confined exclusively to cholinergic junctions. It does not impair the conduction of electrical impulses by nerve or muscle membrane, or the ability of muscle to contract (Burgen *et al.* 1949; Drachman, 1971; Lamanna, 1969), nor does it cause structural damage to nerves or myoneural junctions (Drachman, 1968; Duchen & Strich, 1968; Harris & Miledi, 1971; Thesleff, 1960). Botulinum toxin has been shown not to interfere directly with the activity of the ChE enzyme (Simpson & Morimoto, 1969; Sumyk & Yocum, 1968). Prolonged treatment with botulinum results in typical denervation changes in the morphology, histochemistry

and physiology of skeletal muscle (Drachman, 1964, 1968; Drachman & Houk, 1969; Drachman & Romanul, 1970; Jirmanova, Sobotkova, Thesleff & Zelena, 1964). Evidence that these changes are attributable solely to the cholinergic blockade effect of botulinum toxin has been presented elsewhere (Drachman, 1968). Although the possibility that botulinum toxin might block some specific 'trophic substance' cannot be ruled out, no such action has been demonstrated. Accordingly, the following discussion will be based on our current understanding of the known pharmacological action of botulinum toxin.

Background ChE

Little is presently known about the chemical nature, precise localization, or function of background ChE. By definition, BChE is found in the portion of muscle devoid of end-plates (Guth *et al.* 1964), but whether it is associated with myosin (Varga, Konig, Kiss, Kovacs & Hegedus, 1955) or some other component of the muscle fibre cannot be said at present. It is not certain whether the observed BChE activity is due to a single enzyme species or more than one isozyme. Moreover, its physiological function in muscle is unknown. In the present study, no attempt has been made to resolve these problems, but rather the response of BChE to certain defined treatments has been observed. The results indicate that the BChE responds to botulinum toxin and denervation with a quantitatively identical fall in activity. This suggests that interruption of ACh transmission may fully account for the loss of the neural trophic influence on BChE. It follows that ACh transmission may be necessary for maintenance of the neural trophic influence on BChE under circumstances of normal innervation. However, the precise mechanism by which ACh may influence BChE is not specified by these experimental findings, and remains unknown at present.

End-plate ChE

Considerably more is known about end-plate ChE (EPChE). Functionally, it plays a key role in neuromuscular transmission by hydrolysing released ACh, thereby terminating its transmitter action. EPChE is bound at the synaptic cleft region (Albuquerque, Sokoll, Sonessen & Thesleff, 1968; Hall & Kelly, 1971), predominantly in association with the postjunctional membrane (Barnett, 1962; Lehrer & Ornstein, 1959; Salpeter, 1967). Since less than 10% of the EPChE activity is found within the nerve terminals (Salpeter, 1967), the amount of ChE contained in the nerve is not of significance to the present study.

A large proportion, but not all, of the EPChE activity is under neurotrophic control. As much as 36% of EPChE activity remains 8 weeks after denervation (Guth *et al.* 1964), and histochemical techniques have demon-

strated ChE-positive end-plates 8 months after nerve section (Csillik, 1965). The present study is concerned only with the neurotrophically regulated fraction of the EPChE, and not that which is independent of neural control.

The present results indicate that botulinum toxin and surgical denervation both produce a decrease of EPChE, but the effect of denervation is considerably greater than that of botulinum treatment. This suggests first, that ACh transmission contributes in part to the regulation of EPChE, since there was a significant reduction in EPChE after administration of botulinum toxin. However, since surgical denervation produces almost twice as great a change as does ACh blockade alone, the motor nerve must normally have some influence on EPChE in addition to that of ACh release.

One possible explanation is that the nerve might elaborate a 'trophic factor' in addition to ACh, which contributes in part to the regulation of EPChE. In support of this hypothesis, Lentz (1971) has recently demonstrated that the ChE activity of cultured newt muscle was enhanced by explants of sensory ganglia or extracts of spinal cord, nerves, or liver added to the culture medium. It remains to be shown whether these additives replaced a specific neurotrophic substance missing from the denervated muscle or whether their beneficial effect was of a less specific nature.

An alternative explanation is suggested by recent information which indicates that EPChE is bound to the junctional membrane, from which it can be detached. It has been reported that treatment with proteolytic enzymes results in release of EPChE (Hall & Kelly, 1971), with the concomitant separation of nerve and muscle membrane (Betz & Sakmann, 1971). It is believed that the EPChE is lost as a result of disruption of its presumptive junctional binding sites (Betz & Sakmann, 1971). Denervation also produces separation of nerve and muscle membrane, that is virtually complete by 96–120 hr (Reger, 1959). By contrast, botulinum toxin produces no such change at the neuromuscular junction within the time period of this experiment (Duchen, 1971). The separation caused by denervation, like that due to enzymic treatment, is likely to reflect disruption of the binding sites for ChE at the end-plate. It is tempting to speculate that the greater loss of EPChE following denervation is due to the dual effect of interruption of ACh transmission plus disruption of the presumed ChE binding sites at the end-plate. The partial reduction of EPChE produced by botulinum toxin would be attributed solely to the effect of blockade of ACh transmission.

The significance of the present report is twofold. First, it demonstrates once again that ACh transmission plays an important role in neurotrophic

interactions between nerve and muscle. Secondly, it points to the first instance in which blockade of ACh transmission is not fully equivalent to denervation. In its regulation of EPChE the nerve must exert some trophic action apart from ACh release. It remains to be seen whether a 'trophic substance', the physical contact of nerve and muscle membrane, or some other factor is responsible for this effect.

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