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β-BUNGAROTOXIN STIMULATES THE SYNTHESIS AND ACCUMULATION OF ACETYLCHOLINE IN RAT PHRENIC NERVE DIAPHRAGM PREPARATIONS

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SUMMARY

1. The effects of β-bungarotoxin on acetylcholine (ACh) synthesis, tissue content and release have been studied in the rat diaphragm. A gas chromatographic mass spectrometric assay was used to measure ACh and choline.

2. Within 30 min, β-bungarotoxin (0·14 or 1·4 μg/ml.) caused a significant increase in tissue ACh content. This increase was apparent prior to the final inhibition by β-bungarotoxin of evoked (10 Hz) ACh release.

3. The toxin enhanced the incorporation of $[^2H_4]$Ch into $[^2H_4]$ACh in both resting and stimulated preparations.

4. Hemicholinium-3 blocked the rise in diaphragm ACh normally produced by β-bungarotoxin.

5. β-Bungarotoxin did not directly activate choline acetyltransferase in muscle homogenates.

6. The toxin-induced rise in tissue ACh was largely absent in Ca^{2+}-free solutions which contained either EGTA (1 mM) or $SrCl_2$ (2 or 10 mM).

7. Non-neurotoxic phospholipases A_2, fatty acids and the neurotoxic phospholipase A_2, notexin, did not cause ACh accumulation in the diaphragm.

8. β-Bungarotoxin did not stimulate ACh synthesis in denervated muscle.

9. The extra ACh which accumulated after β-bungarotoxin did not contribute to enhanced release by nerve impulses even when 4-aminopyridine was added to the medium. High K^+ solution and black widow spider venom were also ineffective in increasing output from toxin-treated diaphragms relative to controls that had not been treated with β-bungarotoxin.

10. Prior injection of a rat with botulinum toxin prevented the accumulation of ACh due to β-bungarotoxin. Tubocurarine, however, did not antagonize β-bungarotoxin.

11. These data indicate that β-bungarotoxin has a unique capacity to inhibit ACh release and stimulate ACh synthesis in diaphragm nerve endings. The results are discussed in terms of a possible action of β-bungarotoxin to raise the level of ionized Ca in the nerve terminal cytosol.

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INTRODUCTION

β-Bungarotoxin (β-Btx) is a presynaptically acting polypeptide neurotoxin that is obtained from the venom of the Formosan krait (Chang & Lee, 1963; Chang, Chen & Lee, 1973). The toxin alters cholinergic neuromuscular transmission in a characteristic triphasic sequence (Abe, Limbrick & Miledi, 1976; Abe, Alema & Miledi, 1977; Chang, Su, Lee & Eaker, 1977; Alderdice, 1978; Kelly, von Wedel & Strong, 1979). Following the application of β-Btx, there is an initial period during which the evoked and spontaneous release of acetylcholine (ACh) is inhibited. The first stage gives way to a period of enhanced ACh output. Finally, in the third stage, the inhibition of nerve impulse induced transmitter release is virtually complete.

In 1975 Wernicke, Vanker & Howard reported that a phospholipase A activity was associated with β-Btx. This finding has been repeatedly confirmed (Strong, Goerke, Oberg & Kelly, 1976; Abe *et al.* 1977; Sen, Grantham & Cooper, 1976; Kondo, Toda & Narita, 1978) and has led to numerous hypotheses concerning the relationship of the phospholipase activity of β-Btx to its neurotoxicity. Wernicke *et al.* (1975) contended that the fatty acids liberated by β-Btx cause an uncoupling of nerve terminal mitochondria. The resulting depletion of energy stores was presumed to block ACh release. Alternatively, Sen & Cooper (1978) proposed that β-Btx disrupts membrane phospholipids, thereby depolarizing the nerve ending and inhibiting high affinity choline transport. A reduction of choline uptake would lead to a drop in ACh content and an inhibition of ACh release. A similar disruption of membrane lipids was envisaged by Strong, Heuser & Kelly (1977) as causing an eventual lysis of the axon terminal. More recently, Ng & Howard (1978) proposed that β-Btx-treated synaptosomes respond to membrane depolarization by hydrolyzing ATP in order to restore the membrane potential. A reduction in energy levels was postulated to result in the final blockade of transmitter release caused by β-Btx.

While the foregoing hypotheses account for the eventual cessation of ACh release in β-Btx treated preparations, they do not adequately explain the triphasic nature of the action of the toxin on ACh release. In pursuing this question by chemically measuring the efflux of ACh from toxin-treated rat hemidiaphragms, we observed another effect of β-Btx, namely, a significant rise in ACh content (Gundersen, Newton & Jenden, 1980). Because of the implications of this finding for the mechanism of β-Btx-induced synaptic blockade, as well as for the control of ACh synthesis in the diaphragm, the characteristics of β-Btx action are reported in more detail in this communication.

METHODS

Hemidiaphragm preparations

Male Sprague–Dawley rats (90–150 g) were used. A fan-shaped portion of the left or right hemidiaphragm was dissected at room temperature in an oxygenated Krebs bicarbonate medium, as described by Potter (1970). With the costal margin intact, the wet weight of the entire preparation ranged between 110 and 210 mg, and the diaphragm segment contributed between 50 and 100 mg. Since the wet weight of twenty-four hemidiaphragms dissected from 100 to 150 g rats was 116 ± 8 mg, we routinely used between 40 and 80 % of the available hemidiaphragm for the *in vitro* studies. The average weight of a diaphragm segment was 70 mg.

The tissue was pinned through the rib portion in a temperature-regulated (36–38 °C) organ bath lined with Sylgard (Dow Corning) and containing Krebs solution (3·5 ml.) that was gassed

with 95 % O_2, 5 % CO_2. Routinely, a 30 min equilibration period was used during which time the tissue was exposed to β-Btx (0·14 or 1·4 μg/ml.), eserine, or other agents as noted in the text. The medium was changed once during the equilibration period.

For experiments requiring indirect stimulation, the phrenic nerve was drawn into a suction electrode and stimulated (Grass stimulator, model 509) supramaximally (4–6 V) with 0·1 msec pulses at the frequencies indicated in Results.

To measure released ACh and choline, 3·0 ml. samples of the Krebs solution were removed at the end of the stimulation periods (10 min) or rest periods (15 min). Samples were stored for no more than 90 min in extraction tubes at 4 °C. Each tube contained internal standards for ACh and choline (500 p-mole each of [$^2H_9$]ACh and [$^2H_9$]choline). Between successive stimulation periods 3 min rests intervened. At the end of each experiment the diaphragm was cut free of the costal margin. Both portions of tissue were blotted lightly and placed in separate tubes containing 2·5 ml. 1 N-formic acid in acetone (3:17, v/v) and internal standards for ACh and choline (500 p-mole [$^2H_9$]ACh and [$^2H_9$]choline). The tissue was weighed and ACh and choline were extracted by the procedure given below. In some experiments $^2H_{13}$-labelled compounds were used as internal standards.

Tissue extraction

ACh was quantitatively extracted from the tissue by standing overnight (16–24 hr) in the formic acid/acetone solution at 4 °C. This was confirmed by comparing ACh and choline levels in a series of hemidiaphragms extracted by this procedure with the levels found after homogenizing hemidiaphragms with a homogenizer (Polytron, Brinkman Instruments). Overnight extraction gave ACh and choline levels of 146 ± 5 p-mole (1·25 p-mole/mg wet wt.) and 8·44 ± 0·68 n-mole (69·3 p-mole/mg wet wt.), respectively. Homogenization yielded ACh and choline values of 152 ± 7 p-mole (1·37 p-mole/mg wet wt.) and 6·24 ± 0·62 n-mole (56·3 p-mole/mg wet wt.), respectively. The difference in ACh values is not significant; however, that for choline is significant ($P < 0·05$), suggesting that a small quantity of choline may be released during overnight extractions.

A second test of the overnight extraction procedure involved homogenization of tissues that had been extracted overnight. The additional small quantities of ACh and choline recovered by this procedure (8 and 390 p-mole, respectively) can largely be accounted for by retention of these amines in the fluid spaces of the tissue. Under normal circumstances the inclusion in each sample of internal standards for ACh and choline compensates for this minor loss.

ACh and choline assay

ACh and choline were measured by gas chromatography mass spectrometry (GCMS) using an isotope dilution procedure (Jenden, Roch & Booth, 1973; Freeman, Choi & Jenden, 1975). Briefly, ACh and choline were extracted from aqueous solution at pH 8·5 into dichloromethane containing dipicrylamine (1 mM). The dichloromethane was removed by evaporation. Choline was propionylated and the quaternary amines were demethylated using Na benzenethiolate. After a series of washes, the demethylated products were extracted into dichloromethane and samples were injected into the gas chromatograph. The mass spectrometer was used in the selected ion mode. Quantitation of ACh and choline was achieved by comparing relative signal intensities of the internal standard and unknown at m/e 58 and 64 or 66. The precision of this assay is ± 5 % for samples containing more than 50 p-mole ACh or choline. Moreover, while this work was in progress, several improvements in the sensitivity of the GCMS were obtained. The signal-to-noise ratio that was determined using an injection of 100 p-mole ACh was usually greater than 3000. Thus, the minimum detectable quantity of ACh was in the range of 30 f-mole.

In the experiments in which high levels of [$^2H_4$]choline were added to the Krebs medium, a preliminary treatment was used to remove excess [$^2H_4$]choline. This was done to minimize problems inherent in measuring [$^2H_4$]ACh in the presence of a 10^3–10^4-fold excess of [$^2H_4$]choline. Briefly, choline variants were phosphorylated using choline kinase and ATP by a modification of the procedure of Goldberg & McCaman (1973). The phosphorylcholine remains in the aqueous phase during the initial ion pair extraction procedure that is used in the ACh and choline assay. Under the conditions used, 85 % of the choline was phosphorylated, but problems were encountered in measuring [$^2H_4$]ACh accurately when [$^2H_4$]choline was 100 μM (see Results).

The results for ACh release were normalized to the wet weight of the diaphragm portion of tissue and expressed as f-mole/mg-min. The data for tissue levels of ACh and choline are presented

as p-mole/mg wet wt. of the diaphragm portion. Results are given as mean ± S.E. Significance of the data was evaluated by the paired or unpaired Student's t test, as appropriate.

Twitch tension measurements

A segment of diaphragm tissue was pinned in the bath as described above. A loop of thread was tied through the central tendon and attached to a force displacement transducer (Grass, model FT03C). The signal was amplified and displayed on a recorder (Sanborn, model 320). Experimental procedures are noted in the text.

Choline acetyltransferase assay

A homogenizer (Polytron, Brinkman Instruments) was used to prepare a 1% homogenate of the end plate rich region of rat diaphragm. The composition of the buffer solution and the assay procedure were the same as those described by Fonnum (1975). Corrections were made for blank samples which contained either no eserine or no muscle homogenate. Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951).

Denervation experiments

Animals were anaesthetized with ether or chloralose and the left phrenic nerve was cut within 0·5 cm of the diaphragm. Experiments were performed 5 days post-operatively.

Solutions and chemicals

The normal Krebs solution contained (mM): NaCl, 138; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; KH_2PO_4, 0·4; $NaHCO_3$, 12, and glucose, 11. High K^+ Krebs contained KCl (40 mM) and NaCl was reduced to 103 mM. Ca-free Krebs contained EGTA (1 mM) and no added $CaCl_2$. Sr-containing Krebs had no added Ca and either 2 or 10 mM-$SrCl_2$. Eserine sulphate (15 μM) was added when ACh release was measured.

The β-Btx, notexin and IVa phospholipase employed in these experiments were gifts from Dr Bruce Howard (Department of Biological Chemistry, UCLA School of Medicine). Specifically, the β-Btx component used in this work corresponds to the β_3-Btx of Abe *et al.* (1977) which was obtained by the procedure outlined by Wernicke *et al.* (1975). Botulinum toxin Type A was a gift from Dr E. J. Schantz (University of Wisconsin).

Extracts of venom glands from black widow spiders were produced by homogenizing the glands from one spider in 0·25 ml. 0·9% saline and centrifuging the homogenate at 5×10^3 g for 5 min. The supernatant (50 μl.) was added to the Krebs solution (3·5 ml.) during each 15 min exposure period. We thank Dr W. O. McClure (University of Southern California) for the gift of the black widow spiders.

4-Aminopyridine,(+)-tubocurarine, eserine sulphate, choline kinase, *Vipera russelli* phospholipase A_2, palmitic acid and arachidonic acid were obtained from Sigma Chemical Company (St Louis, MO.). Hemicholinium-3 (Eastman Chemical Company, Rochester, N.Y.) was twice recrystallized from hot ethanol before use.

RESULTS

Effects of β-Btx on diaphragm ACh and choline in vivo

The quantities of ACh and choline were determined in the hemidiaphragms of rats weighing between 100 and 150 g. The right hemidiaphragm usually contained 5–10% more ACh and choline than did the left hemidiaphragm. However, when ACh and choline contents were expressed on a wet weight basis, this small difference disappeared. The first row of Table 1 presents the values for the ACh and choline contents of hemidiaphragms from control animals. These values are similar to those reported by Potter (1970).

When β-Btx (5 μg I.P.) was administered 60 or 90 min before sacrifice, diaphragm ACh content rose to as much as three times that of control (Table 1). Some animals

were in a state of severe respiratory distress 90 min after injection of β-Btx, yet the level of tissue ACh remained higher than control. β-Btx did not significantly alter the tissue choline content (Table 1).

TABLE 1. *In vivo* effects of β-Btx on tissue ACh and choline

Treatment time (min)	*n*	ACh (p-mole/mg)	Choline (p-mole/mg)
0 (control)	32	1·11 ± 0·05	77·7 ± 2·3
60	8	3·10 ± 0·30*	75·6 ± 4·1
90	16	4·17 ± 0·41*	82·7 ± 4·9

The right and left hemidiaphragms were removed from control or toxin-treated rats (5 μg of β-Btx I.P. for indicated time) and the tissue content of ACh and choline were determined. The data are normalized to the wet wt. of the tissue. Values reflect mean ± S.E. of *n* observations.

* Results are significantly different from control at $P < 0·001$.

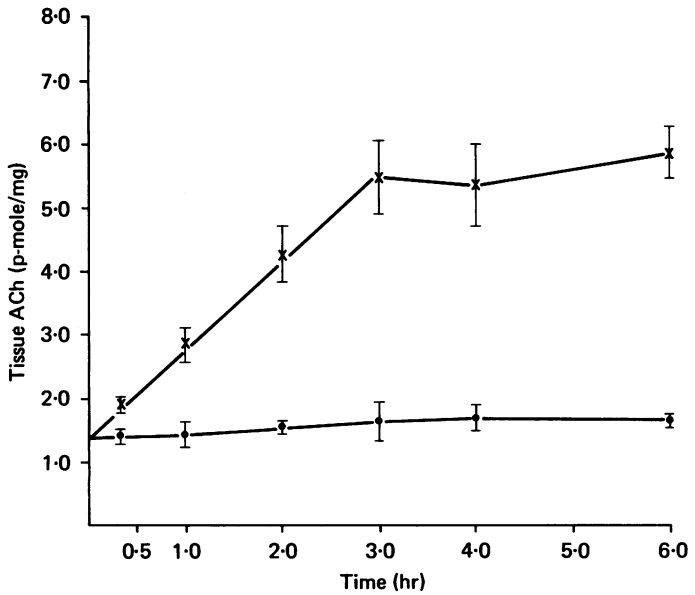

Fig. 1. Effect of β-Btx on tissue ACh in unstimulated preparations. Segments of rat hemidiaphragm were incubated at 37 °C in normal Krebs medium without eserine. Experimental preparations received β-Btx (140 ng/ml.) for the first 30 min of incubation and at the indicated times the quantity of ACh in the experimental and control hemidiaphragms was measured. The results are normalized to the wet wt. of the tissue. Error bars indicate S.E. and *n* ranged between 3 and 9 determinations for each point. Differences between toxin-treated and control are significant at $P < 0·01$ at all times after 30 min.

Time course of β-Btx action on tissue ACh in vitro

The time course of the increase of tissue ACh content caused by β-Btx (140 ng/ml.) was determined in unstimulated preparations with no cholinesterase inhibitor added to the Krebs medium. In most instances both hemidiaphragms from a rat were used, one segment of tissue serving as a control, while a segment of tissue from the other hemidiaphragm was exposed to toxin. β-Btx was added to the medium only during

the first 30 min of incubation. The Krebs solution was changed at 15 min intervals and ACh and choline in the tissue were measured at the times shown in Fig. 1. The ACh content of control hemidiaphragms was very stable. Over a 6 hr period ACh remained at $1{\cdot}5 \pm 0{\cdot}3$ p-mole/mg. The choline content of control tissue (50–85 p-mole/mg) fluctuated more than the ACh content which partly reflects the considerable variation of free choline seen in freshly excised diaphragms (Table 1; and Potter, 1970).

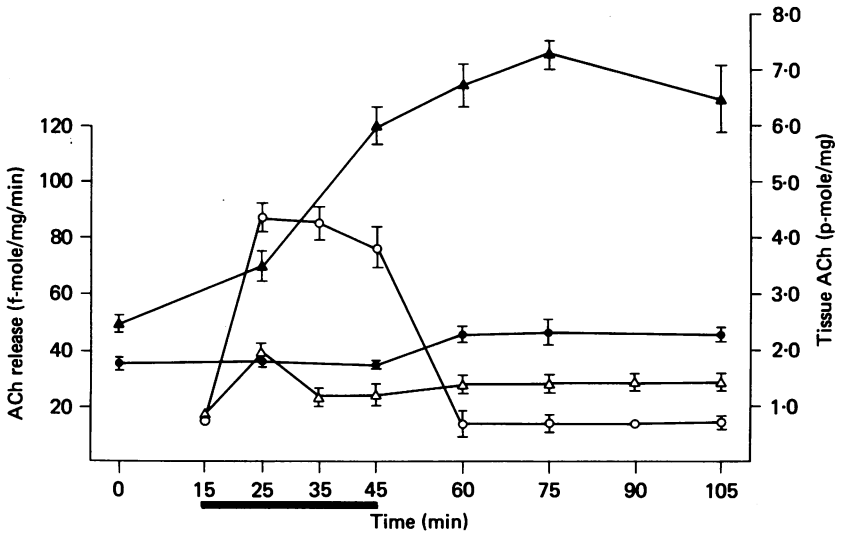

Fig. 2. Effect of β-bungarotoxin on tissue and released ACh in indirectly stimulated preparations. Hemidiaphragms were equilibrated for 30 min with or without β-Btx (1·4 μg/ml.) in a Krebs solution with eserine (15 μM). ACh was measured after successive periods of rest (15 min) or indirect stimulation (10 Hz for 10 min). Stimulation periods are indicated by the filled bar below the abcissa. Tissue ACh was assayed at the times indicated and is normalized to tissue weight. ACh release is normalized to the tissue weight and represents the integrated output of transmitter between successive time points. Each point is the mean ± S.E. of from three to fifteen separate determinations. ○—○ Control: ACh release. ●—● Control: tissue ACh. △—△ β-Btx: ACh release. ▲—▲ β-Btx: tissue ACh.

When hemidiaphragms were incubated for 30 min with β-Btx (140 ng/ml.), a linear increase in tissue ACh was seen during the first 3 hr (Fig. 1), after which ACh content reached a plateau of approximately 5·5 p-mole/mg. The effect of β-Btx on tissue ACh content was thus apparent several hours after washing out the toxin. β-Btx did not have a significant effect on tissue choline content. To minimize inter-animal variation in choline content, segments of right and left hemidiaphragms were taken from the same animal and one segment was exposed to β-Btx. Choline content was measured at the same times indicated in Fig. 1 and the paired Student's t test revealed no significant differences between toxin-treated diaphragms and controls.

Effects of β-Btx on tissue and released ACh in stimulated preparations

In these experiments we correlated the effects of β-Btx on evoked ACh output with its actions on tissue ACh. Diaphragms were exposed to β-Btx (1·4 μg/ml.) during the 30 min equilibration period and after a 15 min rest collection the phrenic nerve

was stimulated (10 Hz) for three consecutive 10 min periods. A series of 15 min rest periods followed the three stimulation periods. Released and tissue ACh were measured at the times indicated in Fig. 2. The normalized output of ACh from controls during 10 min collection periods (Fig. 2) was quite steady in the range of 75–90 f-mole/mg.min. Tissue ACh content was about 1·7 ± 0·1 p-mole/mg after one or three periods of 10 Hz stimulation (Fig. 2). After one 15 min rest following stimulation, tissue ACh had risen to 2·3 ± 0·2 p-mole/mg. This increase is significant at $P < 0·05$ and may be comparable to the rebound rise of tissue ACh that has been reported in superior cervical ganglia of cats (Birks & Fitch, 1974; Bourdois, McCandless & MacIntosh, 1975).

TABLE 2. Effect of β-Btx on [$^2H_4$]choline uptake and [$^2H_4$]ACh synthesis in diaphragm. Indirect stimulation

Condition		Tissue content			
[$^2H_4$]choline	n	[$^2H_0$]ACh (p-mole/mg)	[$^2H_4$]ACh (p-mole/mg)	[$^2H_0$]choline (p-mole/mg)	[$^2H_4$]choline (p-mole/mg)
1 μM					
Control	6	2·14 ± 0·19	0·03 ± 0·03	66·1 ± 6·0	1·2 ± 0·13
β-Btx	5	6·23 ± 0·59**	0·20 ± 0·05*	71·3 ± 12·6	1·7 ± 0·23
10 μM					
Control	5	1·68 ± 0·11	0·94 ± 0·09	65·8 ± 6·9	30·2 ± 2·7
β-Btx	5	4·82 ± 0·43**	3·82 ± 0·42**	70·6 ± 5·0	29·8 ± 2·4
100 μM					
Control	4	0·97 ± 0·11	1·52 ± 0·02	106·3 ± 5·4	323·0 ± 14·8
β-Btx	4	2·37 ± 0·19**	5·70 ± 0·45	115·5 ± 9·8	318·9 ± 35·5

Hemidiaphragms were equilibrated with or without β-Btx (140 ng/ml.) in a Krebs solution with eserine (15 μM) and the indicated concentration of [$^2H_4$]choline. After 30 min the preparation was indirectly stimulated (10 Hz for 10 min) for three successive periods in the eserine-[$^2H_4$]choline containing Krebs. After a final 15 min rest collection the tissue was assayed for all variants of ACh and choline. The results are given as the mean ± S.E.

* Control and toxin-treated differ significantly at $P < 0·01$.

** Control and toxin-treated differ significantly at $P < 0·001$.

After pretreatment with β-Btx (1·4 μg/ml.), ACh release from diaphragms was significantly less than the control rate during all three periods of 10 Hz stimulation (Fig. 2). Tissue ACh content was already significantly higher than control at time 0 (i.e. after the 30 min equilibration period) and approached three times that of control by the end of stimulation (Fig. 2). We did not see a significant decline in tissue ACh content up to 1 hr after stimulation ceased. It is interesting to note that the 'resting' output of ACh from toxin-treated preparations was significantly ($P < 0·05$) greater than release at all times after stimulation stopped (Fig. 2). This may be due to the higher quantity of ACh in the tissue after β-Btx.

Effects of β-Btx on muscle twitch

To obtain an independent assessment of the onset of β-Btx-induced neuromuscular blockade, twitch tension measurements were made on a series of indirectly stimulated hemidiaphragms. Preparations were equilibrated in normal Krebs solution for 30

min. β-Btx (140 ng/ml.) was added at the onset of successive 10 min periods of 10 Hz stimulation. Between each stimulation period there was a 3 min rest during which time the response of muscle to single test pulses was measured. With toxin present, muscle twitch evoked by a single nerve impulse was reduced to 10% of the original tension after 40 ± 3 min. of stimulation ($n = 5$). ACh content of these preparations was $3{\cdot}39 \pm 0{\cdot}15$ p-mole/mg, which is significantly ($P < 0{\cdot}01$) higher than the $1{\cdot}54 \pm 0{\cdot}04$ p-mole ACh/mg found in three control muscles that were stimulated (10 Hz) for a total of 40 min. The relative speed with which β-Btx caused a transmission block might be due to our use of a higher stimulation frequency and thinner tissue preparation than that used by previous investigators (Chang & Lee, 1963; Chang *et al.* 1977).

Effects of β-Btx on ACh synthesis

Three approaches were used in this section. First, the effect of β-Btx on the incorporation of labelled choline into ACh was determined. Secondly, we tested the influence of hemicholinium-3 (HC-3) on the toxin-mediated increase of tissue ACh. Finally, the possibility was considered that β-Btx directly stimulates choline acetyltransferase activity.

Studies employing [$^2H_4$]choline

We used three concentrations of [$^2H_4$]choline, which approximate or exceed the apparent K_T of the high affinity choline carrier in muscle preparations (Vaca & Pilar, 1978), to study the synthesis of ACh in diaphragm. The data in Table 2 provide direct evidence that β-Btx stimulates ACh synthesis. For both toxin-treated and control tissues the level of endogenous and deuterated variants of ACh and choline were measured after three separate periods of indirect stimulation (10 Hz) and a final 15 min rest. As the concentration of [$^2H_4$]choline in the medium was raised, the tissue content of [$^2H_4$]ACh increased (for both control and toxin-treated diaphragms), and the level of [$^2H_0$]ACh declined (Table 2). At all three concentrations of [$^2H_4$]choline, β-Btx caused a significant increase in tissue [$^2H_4$]ACh content relative to the respective control. Moreover, β-Btx increased the tissue [$^2H_0$]ACh content relative to controls, further emphasizing the stimulatory effects of the toxin on ACh accumulation. Since eserine was present to block degradation of ACh by acetylcholinesterase, and as only a small inhibition by β-Btx of total ACh output was observed in these experiments (Table 3), it is evident that the large increase in tissue ACh after β-Btx is due to a stimulation of ACh synthesis.

β-Btx did not significantly affect tissue [$^2H_0$]choline content (Table 2). However, at 1 μM-[$^2H_4$]choline a slight ($P < 0{\cdot}05$) increase of [$^2H_4$]choline accumulation, relative to control, was seen. This effect was not observed at 10 μM or 100 μM-[$^2H_4$]choline, suggesting that β-Btx may enhance a high affinity component of choline transport.

Another effect on choline metabolism, not related to the presence of β-Btx, concerns the higher levels of [$^2H_0$]choline in tissue incubated with 100 μM-[$^2H_4$]choline. Although we were unable to measure accurately the efflux of [$^2H_0$]choline from the tissue when [$^2H_4$]choline was 100 μM, it may be that the high concentration of labelled choline affects the efflux of endogenous choline.

ACh release (Table 3) was measured in the same series of experiments for which

TABLE 3. Effects of β-Btx on [$^2H_0$]ACh and [$^2H_4$]ACh release

Condition		Acetylcholine release (f-mole/mg.min)							
		Period 1		Period 2		Period 3		Period 4	
[$^2H_4$]choline	n	[$^2H_0$]ACh	[$^2H_4$]ACh	[$^2H_0$]ACh	[$^2H_4$]ACh	[$^2H_0$]ACh	[$^2H_4$]ACh	[$^2H_0$]ACh	[$^2H_4$]ACh
1 μM									
Control	6	83 ± 8	3 ± 3	83 ± 14	3 ± 2	76 ± 7	4 ± 4	19 ± 3	2 ± 2
β-Btx	5	84 ± 18	4 ± 3	52 ± 7*	3 ± 2	41 ± 7*	2 ± 2	23 ± 3	1 ± 1
10 μM									
Control	5	62 ± 9	34 ± 15	63 ± 11	49 ± 12	52 ± 7	54 ± 10	20 ± 6	11 ± 6
β-Btx	5	52 ± 8	41 ± 13	44 ± 15	48 ± 11	27 ± 11*	42 ± 6	24 ± 7	22 ± 6
100 μM									
Control	4	79 ± 23	—	52 ± 14	—	33 ± 8	—	25 ± 9	—
β-Btx	4	51 ± 11	—	23 ± 7	—	24 ± 6	—	17 ± 13	—

The results presented in this Table are the quantities of [$^2H_0$]ACh and [$^2H_4$]ACh released during the three periods of 10 Hz stimulation (10 min) and the final 15 min rest (period 4) for the same set of experiments reported in Table 2. Reliable data for [$^2H_4$]ACh were not obtained when [$^2H_4$]choline was 100 μM and these entries were omitted. Otherwise, results are given as the mean ± S.E.

* Control and toxin-treated differ significantly at $P < 0.05$.

the tissue contents were given in Table 2. Although [$^2H_4$]ACh could not be accurately measured in the presence of 100 μM-[$^2H_4$]choline (which amounted to a 10^3-fold excess), data are presented for ACh output in 1 and 10 μM-[$^2H_4$]choline (Table 3). First, it is evident that the amount of [$^2H_4$]choline that was acetylated and then released as [$^2H_4$]ACh increased dramatically as [$^2H_4$]choline was raised from 1 to 10 μM. Secondly, a toxin-induced reduction of ACh output (either [$^2H_0$]ACh or total ACh, [$^2H_0$]ACh + [$^2H_4$]ACh) was not manifest until the second or third period of stimulation. At 1·4 μg/ml. β-Btx caused a significant reduction of ACh release during the first period of 10 Hz stimulation (Fig. 2). Finally, at both 1 and 10 μM-[$^2H_4$]-choline the mole ratio of [$^2H_4$]ACh in the ACh released from control samples during periods 3 and 4 is significantly greater than the corresponding mole ratio in the tissue (calculated from the data in Table 2). This result is consistent with the earlier observation by Potter (1970) of a preferential release of newly synthesized ACh in diaphragm.

Experiments similar to those reported in Tables 2 and 3 were performed but without nerve stimulation (Table 4). Segments of diaphragm were incubated with or without β-Btx (140 ng/ml.) for 30 min in Krebs solution containing eserine (15 μM) and the indicated concentration of [$^2H_4$]choline. After an additional 30 min in the eserine [$^2H_4$]choline Krebs, tissue levels of both variants of ACh and choline were measured.

The data for tissue ACh content in Table 4 are qualitatively similar to those in Table 2, except that ACh content of the stimulated tissue tends to be higher than that found in unstimulated preparations. This may be due to the rebound phenomenon which has been noted previously (Bourdois *et al.* 1975). Additional differences emerge in that β-Btx did not increase [$^2H_4$]ACh synthesis when [$^2H_4$]choline was 1 μM (Table 4), and the [$^2H_0$]ACh content of toxin-treated preparations was not higher than controls at 100 μM-[$^2H_4$]choline (Table 4). More importantly, β-Btx did not demonstrably enhance [$^2H_4$]choline uptake when the labelled choline concentration was 1 μM. This contrasts with the result in Table 2, suggesting that either nerve stimulation or the slightly longer time of exposure to β-Btx (85–90 min in Table 2 *vs.* 60 min in Table 4) might be the underlying factor.

Studies employing hemicholinium-3

In both stimulated and resting preparations hemicholinium-3 (HC-3; 10 μM) completely inhibited the rise of tissue ACh that is associated with β-Btx (Table 5). Moreover, preparations that were stimulated in HC-3-containing Krebs had significantly lower quantities of ACh than those found in unstimulated preparations (compare experiments I and II, Table 5), or in preparations without HC-3 (cf. Fig. 1). This confirms previous reports of the effects of HC-3 on ACh content of diaphragm (Potter, 1970; Gorio, Hurlbut & Ceccarelli, 1978). HC-3 had no apparent effect on the level of tissue choline; the values reported in Table 4 fall within the range given in Table 2. Finally, as reported (Potter, 1970; Gorio *et al.* 1978), the output of ACh into Krebs solutions containing HC-3 declined rapidly during indirect stimulation. By the third period of stimulation (10 Hz), ACh release had fallen to background levels (i.e. 15–20 f-mole/mg. min. β-Btx had no detectable effect on ACh output in these experiments (data not shown). These results imply that HC-3 inhibits the effects of β-Btx on diaphragm ACh via its inhibition of choline uptake (Potter, 1970), though an inhibition of β-Btx binding cannot be excluded.

TABLE 4. Effects of β-Btx on [$^2H_4$]choline uptake and [$^2H_4$]ACh synthesis in diaphragm; unstimulated

Condition		Tissue content			
[$^2H_4$]choline	n	[$^2H_0$]ACh (p-mole/mg)	[$^2H_4$]ACh (p-mole/mg)	[$^2H_0$]choline (p-mole/mg)	[$^2H_4$]choline (p-mole/mg)
1 μM					
Control	5	1·66 ± 0·08	0·10 ± 0·04	53·8 ± 1·7	1·4 ± 0·2
β-Btx	6	2·84 ± 0·12**	0·08 ± 0·05	44·9 ± 3·7	1·4 ± 0·1
10 μM					
Control	3	1·59 ± 0·14	0·43 ± 0·05	54·6 ± 3·7	26·7 ± 1·3
β-Btx	4	2·35 ± 0·24*	1·84 ± 0·05**	50·0 ± 4·0	27·0 ± 1·1
100 μM					
Control	3	1·15 ± 0·11	1·06 ± 0·17	95·0 ± 6·7	262·6 ± 2·3
β-Btx	3	1·55 ± 0·32	2·69 ± 0·28*	93·8 ± 6·1	253·0 ± 11·2

Hemidiaphragms were equilibrated with or without β-Btx (140 ng/ml.) in a Krebs solution with the indicated concentration of [$^2H_4$]choline and eserine (15 μM). Toxin was added for the first 30 min of a 60 min resting incubation after which tissue levels of the variants of ACh and choline were determined. The results are given as the mean ± S.E.

* Control and toxin-treated differ significantly at $P < 0·01$.

** Control and toxin-treated differ significantly at $P < 0·001$.

TABLE 5. Effects of hemicholinium on tissue content of ACh and choline in β-Btx-treated diaphragms

Experiment	n	ACh (p-mole/mg)	Choline (p-mole/mg)
I With stimulation			
Control	3	0·62 ± 0·05	59·6 ± 8·4
β-Btx	3	0·66 ± 0·02	61·8 ± 9·5
II Without stimulation			
Control	4	1·42 ± 0·13	71·5 ± 7·5
β-Btx	4	1·55 ± 0·31	75·0 ± 9·1

Experiment I: hemidiaphragms were equilibrated with or without β-Btx (140 ng/ml.) in a Krebs solution containing hemicholinium-3 (10 μM). After 30 min the muscle was indirectly stimulated (10 Hz) for three successive 10 min periods. Following a 15 min rest, tissue content of ACh and choline was measured.

Experiment II: hemidiaphragms were incubated with or without β-Btx (140 ng/ml.) for 30 min in a Krebs solution containing hemicholinium-3 (10 μM). After an additional 30 min of incubation the tissue content of ACh and choline was determined.

Results are given as the mean ± S.E.

Effects of β-Btx on choline acetyltransferase activity

Two different experiments were performed to test whether β-Btx has a direct influence on diaphragm choline acetyltransferase activity. First, β-Btx (500 ng/ml.) was added to a muscle homogenate for a 15 min preincubation period and the enzyme activity during a subsequent 10 min incubation was compared to control. Under these circumstances the toxin had no effect on muscle choline acetyltransferase activity. Controls synthesized 58 ± 1·5 p-mole ACh/min.mg protein ($n = 3$) and toxin-treated samples synthesized 60 ± 3·1 p-mole ACh/min.mg protein ($n = 3$).

The second protocol involved the measurement of choline acetyltransferase activity in homogenates of diaphragm after the muscles had been incubated for 30 min with β-Btx (140 ng/ml.) in a normal Krebs medium. At this time β-Btx was causing a linear increase of tissue ACh (Fig. 1). Relative to control preparations that had been equilibrated for 30 min in normal Krebs and synthesized 64 ± 4·9 p-mole ACh/min.mg protein, preparations pretreated with β-Btx synthesized 61 ± 3·9 p-mole ACh/min.mg protein. The difference between these two rates of ACh synthesis is not significant, indicating that β-Btx has no detectable effect on choline acetyltransferase activity in muscle homogenates.

TABLE 6. Effects of different agents on diaphragm ACh content

Treatment	*n*	Incubation time (hr)	Tissue ACh content (p-mole/mg)
Series I			
Controls	8	1·0	1·60 ± 0·11
Phospholipase I Va	8	1·0	1·61 ± 0·21
(1·4 μg/ml.)	3	2·0	1·42 ± 0·22
Vipera russelli phospholipase (1·4 μg/ml.)	4	1·0	1·65 ± 0·18
Arachidonic acid (30 μM)	4	1·0	1·69 ± 0·35
Arachidonic acid (100 μM)	4	1·0	1·28 ± 0·08
Palmitic acid (30 μM)	4	1·0	1·63 ± 0·18
Notexin (42 ng/ml.)	7	2·0	0·96 ± 0·07
Series II			
Controls	5	—	1·67 ± 0·16
Calcium free medium	3	—	1·45 ± 0·23
Botulinum toxin	5	—	1·73 ± 0·18

SERIES I: hemidiaphragm segments were incubated in normal Krebs medium with no eserine. The designated agents were added to the medium and after the specified time the tissue ACh was determined. The values reflect the ACh content as normalized to the wet weight of the tissue.

SERIES II: hemidiaphragms were taken from control animals or from rats that had been injected with botulinum toxin (100 LD_{50}) 3 hr before being killed. Tissue segments were equilibrated for 30 min in normal (or Ca^{2+}-free without EGTA) Krebs medium with eserine (15 μM), after which a 15 min rest collection preceded three 10 min periods of indirect stimulation (5 Hz). Tissue ACh was assayed after the final 10 min stimulation period.

Tissue content is given as the mean ± S.E.

Effects of other agents on diaphragm ACh content

Several tests were made of the specificity of the rise in tissue ACh caused by β-Btx. Since β-Btx has phospholipase A_2 activity (Wernicke *et al.* 1975; Strong *et al.* 1976), the diaphragm was treated with other phospholipases A_2 or with fatty acids. Incubation of the muscle preparation with the non-neurotoxic IVa phospholipase A_2 from *Bungarus multicinctus* venom (1 or 2 hr) or with *Vipera russelli* phospholipase A_2 (1 hr) caused no significant change in the level of tissue ACh (Table 6). The concentration of the enzymes employed in these experiments was such that they had 10 to 20 times the phospholipase activity of the β-Btx used in the experiments of Fig. 1, in which a 1 hr exposure to toxin resulted in a doubling of the tissue ACh.

The diaphragm was also incubated with arachidonic acid (30 or 100 μM) or palmitic acid (30 μM) for 1 hr. As the data in Table 6 show, no significant change was produced in the tissue ACh content. These concentrations of fatty acids are capable of reproducing several of the effects of β-Btx in synaptosomes (Wernicke *et al.* 1975).

Like β-Btx, notexin is a presynaptically acting polypeptide neurotoxin with phospholipase A_2 activity (Harris, Karlsson & Thesleff, 1973; Halpert, Eaker & Karlsson, 1976). Unlike β-Btx, notexin caused a significant decline ($P < 0.01$) in diaphragm ACh content relative to controls at 2 hr (compare results in Fig. 1 and Table 6). This observation is consistent with the finding of Dowdall, Fohlman & Eaker (1977) who reported that a similar concentration of notexin inhibited approximately 90% of the high affinity uptake of choline in Torpedo *t* sacs.

A last comment on the data for series 1 (Table 6) concerns the higher ACh content of the control diaphragms relative to that reported for controls in Fig. 1 or Table 1. We have not studied the source of this variability. However, by including controls in each experimental series we eliminated any effects due to differences among groups of animals or in assay conditions.

The data for series 2 (Table 6) show that inhibition of the evoked release of ACh does not, *per se*, cause a rise in diaphragm ACh (Gundersen & Jenden, 1979). Thus, I.P. injection of a rat with botulinum toxin (type A, 100 LD_{50} equivalents for 3 hr) results in a complete inhibition of the evoked release of ACh from diaphragm *in vitro* (C. Gundersen, unpublished observations). Tissue ACh in controls was 1.67 ± 0.16 p-mole/mg, while botulinum toxin-pretreated muscles had 1.73 ± 0.18 p-mole/mg (Table 6). Similarly, omission of Ca^{2+} from the Krebs medium blocked the evoked release of ACh and tissue ACh was slightly lower than control (Table 6).

Effects of divalent cation-modified media on the rise in tissue ACh caused by β-Btx

Calcium ions are required both for the phospholipase activity of β-Btx and for neuromuscular transmission (for discussion see Strong *et al.* 1976). Sr^{2+}, a poor activator of the phospholipase activity of β-Btx (Strong *et al.* 1976), supports a low degree of nerve–muscle transmission (Dodge, Miledi & Rahamimoff, 1969). In these experiments we tested the effects of divalent cation-modified Krebs solutions on ACh release and on the actions of β-Btx.

The rate of ACh release evoked by stimulation (10 Hz) of the phrenic nerve is generally between 60 and 90 f-mole/mg.min for control preparations (Figs. 3–5). The evoked output of ACh in the divalent cation-modified media was significantly depressed relative to these levels of release (Table 5). In fact, in only one instance (10 mM-$SrCl_2$, period 1) was ACh efflux during a stimulation period significantly greater than that observed during a rest period (period 4). These data are consistent with the known Ca^{2+}-dependence of evoked ACh release and with the low efficacy of Sr^{2+} in replacing Ca^{2+} (Dodge *et al.* 1969). Under the conditions of the experiments in Table 5, β-Btx had little additional effect on ACh release. The sole exception was during period 1 in 10 mM-$SrCl_2$ in which β-Btx caused a significant ($P < 0.05$) inhibition of ACh efflux.

The ACh content of control tissue (Table 7) was within the 1·8–2·2 p-mole/mg range observed in diaphragms incubated in normal Krebs medium with eserine (Fig. 2). Thus, neither $SrCl_2$ nor EGTA had any marked effect on tissue ACh content in control preparations. However, these agents effectively blocked the rise of tissue ACh caused by β-Btx in normal Krebs medium (e.g. Fig. 2). Only with 2 mM-$SrCl_2$ did β-Btx promote a significant rise ($P < 0.05$) in diaphragm ACh, though the extent of this increase was less than 15% of that expected in a Ca^{2+}-containing Krebs (see, for example, Fig. 2).

A series of experiments, similar to those reported in Table 7, was performed in which stimulation was omitted. Preparations were bathed in either EGTA (1 mM) or $SrCl_2$ (10 mM) Krebs without eserine. β-Btx (140 ng/ml.) was added to experimental preparations for the first 30 min, and after a 2 hr resting incubation, tissue ACh was measured. Under normal circumstances (e.g. with Ca^{2+} present), treatment

TABLE 7. Effects of β-Btx on released and tissue ACh in divalent cation-modified Krebs solutions

	Treatment	n	Acetylcholine release (f-mole/mg.min) Period 1	Period 2	Period 3	Period 4	Tissue ACh (p-mole/mg)
I	EGTA (1 mM)						
	Control	3	15 ± 2	15 ± 2	12 ± 1	15 ± 2	1·85 ± 0·12
	β-Btx	3	19 ± 10	20 ± 7	16 ± 8	17 ± 5	1·91 ± 0·13
II	$SrCl_2$ (2 mM)						
	Control	3	26 ± 4	21 ± 7	17 ± 2	19 ± 2	2·21 ± 0·22
	β-Btx	4	21 ± 2	23 ± 3	12 ± 2	11 ± 3	2·74 ± 0·06
III	$SrCl_2$ (10 mM)						
	Control	4	30 ± 2	20 ± 2	17 ± 4	17 ± 3	2·09 ± 0·11
	β-Btx	4	23 ± 3	17 ± 2	16 ± 1	13 ± 1	2·08 ± 0·15

Hemidiaphragms were equilibrated with or without β-Btx (140 ng/ml.) in a Ca^{2+}-free Krebs medium supplemented with I: EGTA (1 mM), II: $SrCl_2$ (2 mM), III: $SrCl_2$ (10 nM), and eserine (15 μM). After 30 min there were three 10 min periods of stimulation (10 Hz) and a final 15 min rest collection using the same modified Krebs medium. ACh release was measured and tissue content of ACh was determined at the end of the experiment. Results are given as the mean ± S.E.

with β-Btx leads to a tripling of tissue ACh within 2 hr (see Fig. 1). In divalent cation-modified media this increase of ACh was completely inhibited. Both in EGTA Krebs (four experiments) and in the $SrCl_2$ Krebs (four experiments) tissue stores of ACh remained at the control level of 1·3–1·5 p-mole/mg. These results support the conclusion that Ca^{2+} and perhaps the toxin's phospholipase activity are important to the β-Btx-induced rise in tissue ACh content.

Two additional experiments were performed to test the effects of Ca^{2+} omission on the actions of β-Btx. First, hemidiaphragms were treated with or without β-Btx (140 ng/ml.) in EGTA Krebs, and Ca^{2+} containing Krebs was restored during the subsequent collection periods. Reversing this protocol, muscles were treated with or without toxin in a Ca^{2+}-containing Krebs, and EGTA Krebs was used during the collection periods. Eserine (15 μM) was present at all times. Evoked release of ACh was normal (approximately 60–80 f-mole/mg.min) when Ca^{2+} Krebs was used during the collection periods, but it declined when EGTA Krebs was employed (15–30 f-mole/mg.min). In neither of these experiments did β-Btx affect ACh release.

The data for tissue ACh reveal a significant difference between these two procedures. With EGTA present at the same time as β-Btx, the tissue ACh rose from 1·92 ± 0·07 for controls to 2·92 ± 0·22 p-mole/mg in experiments (n = 4). In contrast, when the Krebs solution contained Ca^{2+} during exposure to β-Btx, tissue ACh increased from 2·11 ± 0·24 to 4·11 ± 0·33 p-mole/mg (n = 3). The rise of tissue ACh effected by β-Btx during the latter experiments is significantly greater ($P < 0·05$) than the

increase caused when the toxin treatment took place in EGTA Krebs. This implies that Ca^{2+} plays an important role in the initiation of action of β-Btx on diaphragm ACh metabolism.

Effects of denervation on the rise of diaphragm ACh content caused by β-Btx

In these experiments the denervated hemidiaphragm was exposed to β-Btx and the contralateral, innervated hemidiaphragm was incubated in normal Krebs without toxin, or alternatively, when the denervated hemidiaphragm was kept in normal Krebs, the innervated hemidiaphragm was treated with β-Btx. All incubations were for 2 hr. β-Btx (140 ng/ml.) was present during the first 30 min only. The protocol was adopted in order to obtain the tissue level of ACh in control preparations (both innervated and denervated) and to ensure that β-Btx retained activity in innervated hemidiaphragms. Thus, we could determine if the toxin was active in a denervated muscle preparation.

TABLE 8. Effects of β-Btx on ACh and choline content of denervated diaphragms

	Treatment	*n*	ACh (p-mole/mg)	Choline (p-mole/mg)
I	Denervated	4	0·18 ± 0·05	39·8 ± 5·7
II	Denervated + β-Btx	5	0·19 ± 0·05	34·6 ± 3·3
III	Innervated	5	1·07 ± 0·08	39·1 ± 3·7
IV	Innervated + β-Btx	4	4·28 ± 0·54	37·5 ± 4·5

Denervated or contralateral, innervated hemidiaphragm segments from rats were treated with or without β-Btx (140 ng/ml.) in a normal Krebs medium. Toxin was added during the first 30 min of a 2 hr resting incubation, after which tissue ACh and choline were determined. Results are presented as the mean ± S.E. of the ACh or choline content normalized to the tissue wet weight.

As the data in Table 8 (row I) indicate, the ACh content of diaphragm segments, 5 days after denervation is approximately 15 % that of innervated controls (row II, Table 8). This quantity is higher than the 2 % of control reported by Potter (1970) and is similar to the 17–21 % of control measured by Hebb, Krnjevic & Silver (1964). Denervation did not change tissue choline content (Table 8; and Potter, 1970).

β-Btx did not significantly affect the amount of ACh in denervated hemidiaphragms (rows I and II, Table 8). However, it did increase ACh in the contralateral, innervated hemidiaphragms (compare rows III and IV, Table 8). Thus, the toxin is active in normally innervated muscle but not in denervated preparations.

Effect of spider venom, high K^+ Krebs and 4-AP on ACh release after β-Btx

A large increase of diaphragm ACh content precedes the final inhibition of evoked transmitter release caused by the toxin (Fig. 2). Thus, it appears that the ACh, which accumulates in these diaphragms, does not lead to a proportionate increase of release by nerve impulses. Black widow spider venom, high K^+ Krebs and 4-aminopyridine (4-AP) were tested for their ability to evoke ACh efflux from β-Btx-treated diaphragms which had accumulated a large store of ACh.

The protocol employed in these experiments was to equilibrate tissue with β-Btx (140 ng/ml.; controls received no toxin) in Krebs solution with eserine (15 μM) for

30 min. The muscle was then stimulated (10 Hz) via the phrenic nerve for three 10 min periods. After a 15 min rest the agent to be tested was applied according to the legend of Fig. 3. In controls that were not treated with β-Btx, spider venom and high K^+ Krebs independently increased the output of ACh relative to the output measured when the agent was not present (that is, resting ACh output in controls is 15 to

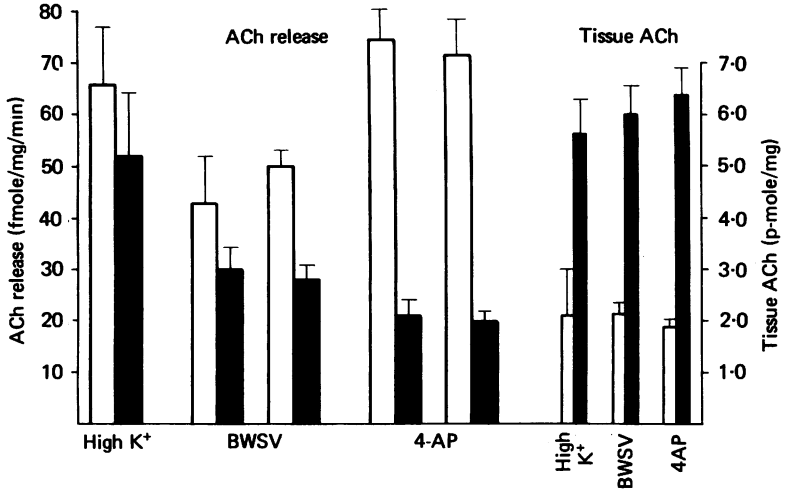

Fig. 3. Effects of high K^+ Krebs, black widow spider venom and 4-aminopyridine on ACh release from β-Btx-treated diaphragms. Diaphragms segments were treated with or with β-Btx (140 ng/ml.) for 30 min in a Krebs medium with eserine (15 μM). After three 10 min periods of indirect stimulation (10 Hz) and a final 15 min rest one of the following treatments was used: 1. High K^+ Krebs: the preparations were treated with high K^+ Krebs, and after one 15 min period, released and tissue ACh content were determined. 2. Spider venom: 50 μl. of an extract of the black widow spider venom gland was added to the Krebs medium at the start of two successive 15 min collection periods. Tissue ACh was measured at the end of the experiment. 3. 4-Aminopyridine: the phrenic nerve was stimulated (10 Hz) for two consecutive periods (10 min) in a Krebs solution containing 4-aminopyridine (100 μM) after which tissue ACh was measured. The left hand portion of the Figure gives the normalized release values and the right hand portion gives the tissue results. All values are mean ± S.E. of at least four separate experiments. The closed bars represent β-Btx treated preparations, while the open bars are controls.

25 f-mole/mg.min; black widow spider venom caused ACh release to reach 40–50 f-mole/mg.min, and high K^+ elicited the release of 60–70 f-mole/mg.min of ACh; see Fig. 3). Similarly, 4-AP (100 μM) increased by 25% the output of ACh during stimulation (10 Hz) of control preparations. Thus, it is evident that these agents stimulate ACh release from control diaphragms. However, as indicated in Fig. 3, not one of these procedures enhanced the output of ACh from β-Btx-treated preparations relative to controls that had not been exposed to β-Btx. Therefore, β-Btx inhibits the release-promoting effect of these agents.

The quantity of ACh in the tissue of control and toxin-treated preparations after challenge with spider venom, high K^+ Krebs or 4-AP is given in the right half of Fig. 3. In all three instances the ACh content of the toxin-treated diaphragms was significantly ($P < 0{\cdot}01$) higher than the level measured in controls without toxin.

It is interesting, however, that we did not observe a large, spider venom-dependent decline in the ACh content of control diaphragms comparable to that reported by Gorio *et al.* (1978) for mouse diaphragms. A possible explanation for this difference in results is the longer time of exposure to this venom in the experiments of Gorio *et al.* (1978).

Tests of botulinum toxin and curare as possible antagonists of the β-Btx-induced rise in tissue ACh content

In preceding sections it has been demonstrated that divalent cation-modified media and HC-3-containing Krebs inhibit the increase in diaphragm ACh caused by β-Btx. We also tested whether botulinum toxin or tubocurarine might inhibit the action of β-Btx. Botulinum toxin was tested because it has been reported to be an antagonist of β-Btx in experiments on muscle twitch response (Chang, Huang & Lee, 1973). In control preparations β-Btx (140 ng/ml.) caused tissue ACh to reach $5{\cdot}06 \pm 0{\cdot}53$ p-mole/mg ($n = 3$) after a 3 hr incubation in normal Krebs. If diaphragms were obtained from rats that were previously injected with botulinum toxin ($100\,LD/_{50}$ I.P., 3 hr), this increase was completely inhibited. ACh content was $1{\cdot}21 \pm 0{\cdot}10$ p-mole/mg ($n = 3$), which is comparable to that ($1{\cdot}36 \pm 0{\cdot}06$ p-mole/mg; $n = 6$) measured in diaphragms from rats injected with botulinum toxin ($100\,LD/_{50}$, 3 hr) and not exposed to β-Btx. A similar antagonism of β-Btx by botulinum toxin (administered I.P.) has been observed in preparations receiving indirect stimulation after exposure to both toxins (C. Gundersen & M. Newton, unpublished observations). Thus, botulinum toxin appears to inhibit either the binding or the subsequent action of β-Btx, thereby preventing the accumulation of ACh.

Because it inhibits the stimulation-dependent rise in ACh content of sympathetic ganglia (Bourdois *et al.* 1975), tubocurarine was tested for its ability to inhibit the effect of β-Btx on diaphragm ACh content. Preparations were treated the same way as those described in the legend to Table 2, except that tubocurarine (10 μM) replaced [$^2H_4$]choline. Tissue content of ACh in controls after the final 15 min rest period were $1{\cdot}96 \pm 0{\cdot}07$ p-mole/mg. Preparations that had received β-Btx (140 ng/ml.) contained $6{\cdot}73 \pm 1{\cdot}01$ p-mole/mg of ACh, which indicates that curare did not significantly inhibit the rise in tissue ACh caused by β-Btx.

DISCUSSION

The data presented in this paper confirm and extend our preliminary observations (Gundersen *et al.* 1980) of a simultaneous inhibition of evoked transmitter release and an accumulation of ACh in rat phrenic nerve-diaphragm preparations treated with β-Btx. Using [$^2H_4$]choline we obtained direct evidence of a stimulation by β-Btx of [$^2H_4$]ACh synthesis (Table 2). However, β-Btx did not detectably enhance the activity of choline acetyltransferase that was measured in muscle homogenates. Rather, the toxin appeared to interfere with the regulation of ACh synthesis while concomitantly depressing the evoked release of ACh and eliminating muscle twitch. We will discuss the possibility that these correlates are due to a toxin-mediated increase of Ca^{2+} in the nerve terminal cytosol.

A substantial body of evidence suggests that β-Btx affects the concentration or

movement of Ca^{2+} in excitable cells (Kelly & Brown, 1974; Lau, Chiu, Caswell & Potter, 1974; Wagner, Mart & Kelly, 1974; Wernicke *et al.* 1975; Abe *et al.* 1976; Oberg & Kelly, 1976; Swanson, 1977; Ng & Howard, 1979). Although it remains to be shown by direct means that β-Btx causes a sustained increase of cytosolic Ca^{2+}, the triphasic effects of β-Btx on ACh release (Abe *et al.* 1976) and the toxin's stimulation of ACh synthesis might develop in the following manner. The early, phospholipase-independent inhibition of ACh release is probably correlated with toxin binding (Abe *et al.* 1976; Kelly *et al.* 1979); then the toxin-mediated depolarization of nerve endings (Sen & Cooper, 1978; Ng & Howard, 1979) initiates the rise of nerve terminal Ca^{2+} leading to the period of enhanced transmitter release. We propose that this second phase begins the period of enhanced synthesis and accumulation of tissue ACh. As the Ca^{2+} concentration continues to rise, the quantal release mechanism becomes insensitive to the release-triggering action of Ca^{2+}. A blockade of evoked ACh release ensues, and, more slowly, ACh content plateaus at approximately three times the control level.

We will discuss in more detail two aspects of this model. First, the regulatory mechanism for ACh synthesis in motor nerve terminals has not been determined. Several possibilities have been discussed (e.g. mass action, end-product inhibition, substrate availability or the activity of choline acetyltransferase; see MacIntosh & Collier, 1976, for a review) and if our hypothesis is correct, then Ca^{2+} must act on one of these processes. Vaca & Pilar (1978) have presented evidence for a role of Ca^{2+} in modulating ACh synthesis. They propose that the Ca^{2+}-dependent release of ACh reduces nerve-terminal ACh which promotes ACh synthesis via a mass action effect on choline acetyltransferase. Our postulate anticipates a more direct role of Ca^{2+}, perhaps in the translocation of ACh into synaptic vesicles or in the supply of acetyl-CoA or choline to the enzyme. Future investigations using β-Btx should be valuable for discriminating among these alternatives.

The second comment concerns precedents for the inhibition of quantal ACh release by high Ca^{2+} in the nerve ending. Several different substances, including ouabain, the ionophore X-537A and mersalyl, produce a transient, massive increase in the frequency of miniature end plate potentials which is followed by a virtually complete inhibition of quantal events (Birks & Cohen, 1968; Kita & van der Kloot, 1976; Binah, Meiri & Rahamimoff, 1978). Since it has been proposed that these agents lead to an accumulation of Ca^{2+} in the nerve terminal, it may be that excessive quantities of this cation inhibit ACh release.

β-Btx stimulates that component of choline transport which supplies precursor for ACh synthesis. This is attested to by the increased synthesis of [$^2H_4$]ACh (Table 2) and by the depressant effect of HC-3 (Table 5), an inhibitor of choline uptake (Potter, 1970), on ACh accumulation after β-Btx. A kinetic investigation of these findings was precluded by two considerations. First, diaphragms contain (Table 1) and release (efflux rates of choline from diaphragm segments exceed 100 p-mole/min) large quantities of choline. This makes it difficult to estimate with accuracy the concentration of labelled choline at diaphragm nerve endings within the time periods employed in measurements of the initial rate of transport. The second complication arises in obtaining a viable means of distinguishing between neuronal and non-neuronal uptake of choline in muscle preparations. Suszkiw & Pilar (1977) used denervated muscle

as an index of the non-neuronal uptake of choline. This approach ignores possible changes of choline transport in denervated muscle. Reductions in acetylcholinesterase activity, changes in ACh receptor distribution and a decline in resting membrane potential are well known consequences of denervation (Thesleff, 1974; Gutman, 1976). A reduction of membrane potential could have a significant effect on choline uptake, as it would reduce the electrochemical gradient for choline accumulation. Jope (1980) reviews the general problem of devising meaningful estimates of non-specific choline uptake. In spite of these difficulties, experiments are currently in progress to determine whether β-Btx has a direct effect on the coupling of choline transport to ACh synthesis.

The prevention by divalent cation-modified media of the β-Btx-induced increase of tissue ACh in stimulated preparations (Table 7) is consistent with the proposal (Wernicke *et al.* 1976; Strong *et al.* 1976; Abe *et al.* 1977) that the phospholipase activity of β-Btx is involved in its neurotoxicity. The normal increase in tissue ACh content of toxin-treated preparations in 2 mM-$SrCl_2$ (Table 7) may be due to the residual phospholipase activity of the toxin in 2 mM-$SrCl_2$ (Strong *et al.* 1976; Chang *et al.* 1977).

Restoring Ca^{2+} to the medium of diaphragms that were pretreated with β-Btx in EGTA Krebs resulted in a 50% increase of tissue ACh relative to control. The complementary procedure (pretreat with β-Btx in Ca^{2+}-containing Krebs and then switch to EGTA Krebs) resulted in a significantly greater ($P < 0{\cdot}05$) increase of tissue ACh to twice the control level. These results suggest that the binding of β-Btx or an early stage of the toxin's action is suppressed in EGTA Krebs. Moreover, a 30 min exposure of diaphragms to β-Btx in normal Krebs causes approximately a 50% increase of tissue ACh (Fig. 1). However, subsequent incubation of these diaphragms in EGTA-containing Krebs results in the ACh content (as noted above) approaching twice the control level. Thus, it appears that either β-Btx or some consequence of the toxin's action, continues to affect ACh synthesis despite the reduction of medium Ca^{2+}.

We tested a number of agents and procedures for their ability to mimic the effect of β-Btx on tissue ACh. First, we investigated the possibility that the increase of tissue ACh content was a primary consequence of a β-Btx-mediated inhibition of transmitter release. Evoked ACh release was blocked by pretreating rats with botulinum toxin or by incubating the tissue in Ca^{2+}-free medium. The data in Table 6 indicate that tissue ACh did not increase as a consequence of either of these treatments. Thus, inhibition of evoked transmitter release by β-Btx does not *per se* lead to a rise of tissue ACh. This conclusion is strengthened by the fact that β-Btx inincreased the ACh content of unstimulated preparations. Moreover, the net increase of tissue ACh after β-Btx is greater than one can account for by a complete inhibition of ACh release.

Another approach that we used to attempt to reproduce the action of β-Btx took advantage of the fact that the toxin has phospholipase A_2 activity (Wernicke *et al.* 1975). Both the IVa phospholipase A_2 from *Bungarus multicinctus* venom and that from *Vipera russelli* venom were found to be without significant effect on tissue ACh content. The same holds true for the fatty acids, arachidonic and palmitic, neither of which caused ACh accumulation when used in concentrations that uncouple

mitochondria and inhibit synaptosomal γ-aminobutyric acid transport (Wernicke *et al.* 1975). These results imply that either the phospholipase activity of β-Btx is not involved in raising the tissue ACh content or that the toxin has unique binding properties or substrate specificity which distinguish its actions from those of the foregoing procedures. A similar argument was presented by Ng & Howard (1978) and Kelly *et al.* (1979) to explain the neurotoxicity of β-Btx.

In contrast to β-Btx, notexin decreased the ACh content of diaphragms (Table 6). Notexin, a presynaptically acting neurotoxin with phospholipase A_2 activity (Halpert *et al.* 1976), has a triphasic effect on transmitter release at frog neuromuscular junctions that is similar to that of β-Btx (Magazanik & Slavnova, 1978). Moreover, notexin inhibits by more than 90 % the high affinity uptake of [^{3}H]choline by Torpedo *t* sacs (Dowdall *et al.* 1977). If these actions of notexin were present at diaphragm nerve endings, it could explain the decline of ACh content that we observed. A paradox arises in that β-Btx has also been reported to inhibit the high affinity transport of [^{3}H]choline by *t* sacs (Dowdall *et al.* 1977) and by rat brain P_2 fractions (Sen *et al.* 1976). The problem of how β-Btx could inhibit choline uptake in these preparations and cause an apparent stimulation of choline uptake and ACh synthesis in diaphragm seems to have been partially resolved by a recent report (Dowdall *et al.* 1979) in which β-Btx had no detectable effect on choline transport in purified synaptosomal preparations. Nevertheless, it should be of considerable interest to resolve the difference in mode of action of notexin and β-Btx in diaphragm.

The ACh content of denervated muscle was unaffected by β-Btx, while treatment of the contralateral, innervated hemidiaphragm with β-Btx resulted in a normal increase of tissue ACh (Table 8). These findings argue that the actions of β-Btx are restricted to neuronal elements. However, it cannot be excluded that other concomitants of denervation (for a discussion of these changes see Thesleff, 1974) inhibit the effect of the toxin on tissue ACh metabolism.

Previous investigations, which have used bioassays of the ACh released from diaphragm preparations, have shown that both high K^+ solutions and extracts of the black widow spider venom gland stimulate the efflux of ACh (Mitchell & Silver, 1964; Gorio *et al.* 1978). We confirmed these results (Fig. 3) and also demonstrated that 4-AP (100 μM) enhances the nerve impulse-induced output of ACh from controls. Yet none of the selected agents enhanced the release of ACh from toxin-treated preparations relative to control (Fig. 3). Since these agents have been postulated to enhance ACh release by increasing the intraterminal Ca^{2+} concentration (Lundh, Leander & Thesleff, 1977; Simpson, 1978), their failure to overcome the β-Btx-mediated blockade of ACh release is consistent with β-Btx having caused an accumulation of Ca^{2+} in the nerve ending.

Tubocurarine did not antagonize the β-Btx-induced rise of tissue ACh. This provides a pharmacological distinction between the toxin effect and the rebound rise of ganglionic ACh which is blocked by tubocurarine (Bourdois *et al.* 1975). However, botulinum toxin inhibited the effect of β-Btx on tissue ACh. Since the inhibition by botulinum toxin on ACh release in rat muscle has been attributed to a reduced sensitivity to Ca^{2+} of a component of the transmitter release process (Lundh *et al.* 1977), it may be that botulinum toxin also effects a reduction of the postulated sensitivity to Ca^{2+} of the ACh synthetic machinery in nerve endings. Thus, botulinum

toxin would simultaneously block the effects on ACh release and synthesis of the rise in cytosolic Ca^{2+} concentration which we have postulated to underlie the action of β-Btx.

Chang *et al.* (1973) did not report a significant effect of β-Btx on diaphragm ACh in experiments using long-term treatment with β-Btx. Perhaps the most likely explanation for the difference in results is the acute denervation-like syndrome that has been observed in muscles treated for long periods with β-Btx (Chang, Chuang & Huang, 1975; Abe *et al.* 1976). Denervation reduces the ACh content of muscle (Table 8) and recent experiments (Gundersen & Newton, unpublished observations) indicate that sustained exposure to β-Btx (1·4 μg/ml. for 3 hr) largely inhibits the increase of tissue ACh that is otherwise measured (Figs. 1 and 2).

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REFERENCES

ABE, T., LIMBRICK, A. R. & MILEDI, R. (1976). Acute muscle denervation induced by β-bungarotoxin. *Proc. R. Soc.* B. **194**, 545–553.

ABE, T., ALEMA, S. & MILEDI, R. (1977). Isolation and characterization of presynaptically acting neurotoxins from the venom of Bungarus snakes. *Eur. J. Biochem.* **80**, 1–12.

ALDERDICE, M. T. (1978). An early decrease in transmitter release produced by β-bungarotoxin. *Fedn. Proc.* **37**, 149.

BINAH, O., MEIRI, V. & RAHAMIMOFF, H. (1978). The effects of $HgCl_2$ and mersalyl on mechanisms regulating intracellular calcium and transmitter release. *Eur. J. Pharmacol.* **51**, 453–457.

BIRKS, R. I. & COHEN, M. W. (1968). The action of sodium pump inhibitors on neuromuscular transmission. *Proc. R. Soc.* B. **170**, 384–399.

BIRKS, R. I. & FITCH, S. J. G. (1974). Storage and release of acetylcholine in a sympathetic ganglion. *J. Physiol.* **240**, 125–134.

BOURDOIS, P. S., MCCANDLESS, D. L. & MACINTOSH, F. C. (1975). A profound after effect of intense synaptic activity on acetylcholine in a sympathetic ganglion. *Can. J. Physiol. Pharmacol.* **53**, 155–165.

CHANG, C. C. & LEE, C. Y. (1963). Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Archs int. Pharmacol. Thér.* **144**, 241–257.

CHANG, C. C., CHUANG, S. T. & HUANG, M. C. (1975). Effects of chronic treatment with various neuromuscular blocking agents on the number and distribution of acetylcholine receptors in the rat diaphragm. *J. Physiol.* **250**, 161–173.

CHANG, C. C., CHEN, T. F. & LEE, C. Y. (1973). Studies of the presynaptic effect of β-bungarotoxin on neuromuscular transmission. *J. Pharmac. exp. Ther.* **184**, 339–345.

CHANG, C. C., SU, M. J., LEE, J. D. & EAKER, D. (1977). Effects of Sr^{2+} and Mg^{2+} on the phospholipase A and presynaptic neuromuscular blocking actions of β-bungarotoxin, crotoxin and taipoxin. *N.S. Arch. Pharmacol.* **299**, 155–161.

DODGE, F. A., MILEDI, R. & RAHAMIMOFF, R. (1969). Strontium and quantal release of transmitter at the neuromuscular junction. *J. Physiol.* **200**, 267–283.

DOWDALL, M. J., FOHLMAN, J. & EAKER, D. (1977). Inhibition of high affinity choline transport in peripheral cholinergic endings by presynaptic snake venom neurotoxins. *Nature, Lond.* **269**, 700–702.

DOWDALL, M. J., FOHLMAN, J. & WATTS, A. (1979). Presynaptic action of snake venom neurotoxins on cholinergic systems. *Adv. Cytopharmacol.* **3**, 63–76.

FONNUM, F. (1975). A rapid radiochemical method for the determination of choline acetyltransferase. *J. Neurochem.* **24**, 407–409.

FREEMAN, J. J., CHOI, L. & JENDEN, D. J. (1975). Plasma choline: its turnover and exchange with brain choline. *J. Neurochem.* **24**, 729–734.

GOLDBERG, A. M. & MCCAMAN, R. E. (1973). The determination of picomole amounts of acetylcholine in mammalian brain. *J. Neurochem.* **20**, 1–8.

GORIO, A., HURLBUT, W. P. & CECCARELLI, B. (1978). Acetylcholine compartments in mouse diaphragm. *J. cell. Biol.* **78**, 716–733.

GUNDERSEN, C. B. & JENDEN, D. J. (1979). Botulinum toxin depresses the resting output of acetylcholine from the rat diaphragm. *Trans. Am. Soc. Neurochem.* **10**, 119.

GUNDERSEN, C. B., NEWTON, M. W. & JENDEN, D. J. (1980). β-Bungarotoxin elevates acetylcholine levels in rat diaphragm. *Brain Res.* **182**, 486–490.

GUTMANN, E. (1976). Neurotrophic relations. *A. Rev. Physiol.* **38**, 177–216.

HARRIS, A. J. & MILEDI, R. (1971). Effect of Type D botulinum toxin on frog neuromuscular junctions. *J. Physiol.* **217**, 497–515.

HARRIS, J. B., KARLSSON, E. & THESLEFF, S. (1973). Effects of an isolated toxin from Australian Tiger snake (*Notechis scututas scutatus*) venom on the mammalian neuromuscular junction. *Br. J. Pharmac. Chemother.* **47**, 141–146.

HALPERT, J., EAKER, D. & KARLSSON, E. (1976). The role of phospholipase activity in the action of a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian Tiger snake). *FEBS Lett.* **61**, 72–76.

HEBB, C. O., KRNJEVIC, K. & SILVER, A. (1964). Acetylcholine and choline acetyl-transferase in the diaphragm of the rat. *J. Physiol.* **171**, 504–513.

JENDEN, D. J., ROCH, M. & BOOTH, R. B. (1973). Simultaneous measurement of endogenous and deuterium labelled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography mass spectrometry. *Analyt. Biochem.* **55**, 438–448.

JOPE, R. S. (1980). High affinity choline transport and acetyl CoA production in brain and their roles in the regulation of acetylcholine synthesis. *Brain Res. Rev.* **1**, 313–344.

KAO, I., DRACHMAN, D. B. & PRICE, D. L. (1977). Botulinum toxin: mechanism of presynaptic blockade. *Science, N.Y.* **193**, 1256–1258.

KELLY, R. B. & BROWN, F. R. (1974). Biochemical and physiological properties of a purified snake venom neurotoxin which acts presynaptically. *J. Neurobiol.* **5**, 135–150.

KELLY, R. B., VON WEDEL, R. J. & STRONG, P. N. (1979). Phospholipase dependent and phospholipase independent inhibition of transmitter release by β-bungarotoxin. *Adv. Cytopharmacol.* **3**, 77–84.

KITA, H. & VAN DER KLOOT, W. (1976). Effects of the ionophore X-537 A on acetylcholine release at the frog neuromuscular junction. *J. Physiol.* **259**, 177–198.

KONDO, K., TODA, H. & NARITA, K. (1978). Characterization of phospholipase A activity of β_1-bungarotoxin from *Bungarus multicinctus* venom. I. Its enzymatic properties and modification with p-bromphenacyl bromide. *J. Biochem. (Tokyo)* **84**, 1291–1300.

LAU, M. H., Chiu, T. H., Caswell, A. H. & Potter, L. T. (1974). Effects of β-bungarotoxin on calcium uptake by sarcoplasmic reticulum from rabbit skeletal muscle. *Biochem. biophys. Res. Commun.* **61**, 510–516.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

LUNDH, H., LEANDER, S. & THESLEFF, S. (1977). Antagonism of the paralysis produced by botulinum toxin in the rat. *J. neurol. Sci.* **32**, 29–43.

MACINTOSH, F. C. & COLLIER, B. (1976). Neurochemistry of cholinergic terminals. In *Neuromuscular Junction*, ed. ZAIMIS, E., pp. 88–228. New York: Springer-Verlag.

MAGAZANIK, L. G. & SLAVNOVA, T. I. (1978). Effects of presynaptic polypeptide neurotoxins from tiger snake venom (notechis-II-5 and notexin) on frog neuromuscular junction. *Physiologia bohemoslov.* **17**, 421–429.

MITCHELL, J. F. & SILVER, A. (1974). The spontaneous release of acetylcholine from the denervated hemidiaphragm of the rat. *J. Physiol.* **165**, 117–129.

NG, R. & HOWARD, B. D. (1978). De-energization of nerve terminals by β-bungarotoxin. *Biochemistry, N.Y.* **17**, 4978–4986.

NG, R. & HOWARD, B. D. (1980). Mitochondria and sarcoplasmic reticulum as model targets for neurotoxic and myotoxic phospholipases A_2. *Proc. natn. Acad. Sci. U.S.A.* (In the Press.)

OBERG, S. G. & KELLY, R. B. (1976). The mechanism of β-bungarotoxin action. I. Modification of transmitter release at the neuromuscular junction. *J. Neurobiol.* **7**, 129–141.

POTTER, L. T. (1970). Synthesis, storage and release of [^{14}C]acetylcholine in isolated rat diaphragm muscles. *J. Physiol.* **206**, 145–166.

PUMPLIN, D. W. & DEL CASTILLO, J. (1975). Release of packets of acetylcholine and synaptic vesicles by brown widow spider venom in frog motor nerve endings poisoned by botulinum toxin. *Life Sci. Oxford.* **17**, 137–142.

SEN, I. & COOPER, J. R. (1978). Similarities of β-bungarotoxin and phospholipase A_2 and their mechanisms of action. *J. Neurochem.* **30**, 1369–1375.

SEN, I., GRANTHAM, P. A. & COOPER, J. R. (1976). Mechanism of action of β-bungarotoxin on synaptosomal preparations. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2664–2668.

SIMPSON, L. L. (1978). Pharmacological studies on the subcellular site of action of botulinum toxin type A. *J. Pharmac. exp. Ther.* **206**, 661–669.

STRONG, P. N., GOERKE, J., OBERG, S. G. & KELLY, R. B. (1976). β-Bungarotoxin, a presynaptic toxin with enzymatic activity. *Proc. natn. Acad. Sci. U.S.A.* **73**, 178–182.

STRONG, P. N., HEUSER, J. E. & KELLY, R. B. (1977). Selective enzymatic hydrolysis of nerve terminal phospholipids by β-bungarotoxin: biochemical and morphological studies. In *Cellular Neurobiology*, ed. Z. HALL & C. F. FOX, pp. 227–249. New York: A. R. Liss.

SUSZKIW, J. B. & PILAR, G. (1976). Selective localization of a high affinity choline uptake system and its role in ACh formation in cholinergic nerve terminals. *J. Neurochem.* **26**, 1133–1138.

SWANSON, P. D. (1977). Effects of β-bungarotoxin, diphenylhydantoin and metabolic inhibitors on calcium uptake and on monovalent cations and high-energy phosphate contents of brain slices. *J. Neurochem.* **29**, 767–769.

THESLEFF, S. (1974). Physiological effects of denervation of muscle. *Ann. N.Y. Acad. Sci.* **228**, 89–104.

VACA, K. & PILAR, G. (1979). Mechanisms controlling choline transport and acetylcholine synthesis in motor nerve terminals during electrical stimulation. *J. gen. Physiol.* **78**, 605–628.

WAGNER, G. M., MART, P. E. & KELLY, R. B. (1974). β-Bungarotoxin inhibition of calcium accumulation by rat brain mitochondria. *Biochem. biophys. Res. Commun.* **58**, 475–481.

WERNICKE, J. F., VANKER, A. D. & HOWARD, B. D. (1975). The mechanism of action of β-bungarotoxin. *J. Neurochem.* **25**, 483–496.