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Mechanism of action of β-bungarotoxin on synaptosomal preparations

(neurotoxin/acetylcholine/high-affinity choline uptake)

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ABSTRACT **The neurochemical activity of β-bungarotoxin was investigated using a synaptosomal preparation of rat cerebral cortices. In preparations preincubated with [^{3}H]choline in order to label acetylcholine the toxin caused a rapid release of the transmitter, which was calcium dependent but only a little affected by a depolarizing concentration of potassium. β-Bungarotoxin was also shown to be a potent inhibitor of the high-affinity transport system for choline, producing 50% inhibition at a concentration of 50 nM. These findings explain the observed electrophysiological effects of the toxin. Electron microscopy revealed no discernible effect of 0.1 μM β-bungarotoxin on either synaptic vesicles or mitochondria. Neither the release of transmitter nor the inhibition of choline uptake by the toxin was affected by the presence of an inhibitor of phospholipase activity.**

The neuromuscular blocking action of the venom of *Bungarus multicinctus* has been recently studied in detail. The crude venom can be separated into four different fractions, the α-, β-, and γ-bungarotoxins, and cholinesterase (1). α-Bungarotoxin produces a postsynaptic neuromuscular blockade by acting on the nicotinic acetylcholine receptors (1, 2), whereas β- and γ-bungarotoxins, which also block neuromuscular transmission, appear to act exclusively on the presynaptic side of the neuromuscular junction (1, 3, 4).

Rat phrenic nerve–diaphragm preparations were used by Chang *et al.* (5) to study the blocking effect of β-bungarotoxin (β-BuTX). Initially the toxin increased the frequency of miniature endplate potentials followed by a decreased output and, finally, complete inhibition of nerve impulse-induced release of acetylcholine (AcCh) leading to a neuromuscular block (5). According to Chen and Lee (6) electron microscopic studies revealed that an injection of β-bungarotoxin produced a depletion of synaptic vesicles, whereas Kelly and Brown (7) observed an accumulation of "coated vesicles" in toxin-treated terminals rather than open synaptic vesicles. A swelling of mitochondria in the axon terminals has also been observed under the electron microscope (6).

Recently it was reported that β-bungarotoxin is also active on mammalian brain synapses, resulting in an efflux of γ-aminobutyric acid, norepinephrine, and serotonin (8), as well as 2-deoxyglucose (9). These authors suggest that β-bungarotoxin acts by virtue of its endogenous phospholipase activity, which inhibits oxidative phosphorylation in the mitochondria of nerve terminals.

The present communication describes neurochemical studies on the direct effect of β-bungarotoxin on rat cortical synaptosomal enriched fractions on the release of AcCh, and kinetics of the inhibition of uptake of choline. Part of this study has been presented in a preliminary communication (10).

MATERIALS AND METHODS

The β-bungarotoxin used in this study, kindly provided by Dr. Jean Rosenthal, was purified from the crude venom of *Bungarus multicinctus* according to the procedure of Lee *et al.* (11). Fraction V, molecular weight 28,500, was used in all studies. Choline chloride was purchased from Sigma Chemical Co. Acetylcholine chloride was supplied by Matheson, Coleman and Bell. Nutritional Biochemical Corp. was the source of eserine sulfate. *S*-Acetylthiocholine iodide was purchased from Calbiochem. [*Methyl*-^{3}H]choline, specific activity 10.1 Ci/mmol, was obtained from Radiochemical Centre, Amersham.

Preparation of Synaptosomes. The cerebral cortices of male, 21- to 30-day-old rats were dissected out rapidly after decapitation, as much white matter as possible was removed, and the remaining gray matter was homogenized in 0.32 M sucrose containing 2 mM Tris·HCl at pH 7.4 and 40 μM EDTA. The "P_2" fraction was prepared according to the method of Cotman (12) and washed twice before use. Under the electron microscope this fraction was judged to be approximately 70% synaptosomal.

Release of AcCh from Synaptosomes. The P_2 fraction was loaded with labeled AcCh by a preincubation of 30 min at 37° with [*methyl*-^{3}H]choline, specific activity 0.35 Ci/mmol, final concentration 9.4 μM, in Krebs–Ringer bicarbonate medium (KR), gassed with 95% O_2–5% CO_2. This medium had a final concentration of 5.6 mM KCl, 139 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 11 mM $NaHCO_3$, and 1 mM NaH_2PO_4. After preincubation, the suspension was kept in ice for 5 min and centrifuged at 15,000 × *g* for 10 min. The supernatant was discarded and the pellet was washed once with an equal volume of KR containing 0.2 mM eserine sulfate. The P_2 fraction was resuspended in KR containing eserine and incubated at 37° in normal KR (5.3 mM K^+) or in KR containing a high KCl concentration (53 mM) with or without various concentrations of β-bungarotoxin. After 20 min, the tubes were transferred to ice for 5 min and centrifuged rapidly, and the supernatant was diluted 1:1 with 1 M formic acid/acetone (1:3 vol/vol) and centrifuged. An aliquot of the supernatant, to which unlabeled carrier AcCh and choline were added, was subjected to high voltage electrophoresis to separate AcCh from choline (13). The AcCh spot was cut out, eluted, and the radioactivity was determined using Aquasol scintillation liquid. Protein was measured by the procedure of Lowry *et al.* (14) using bovine serum albumin as standard.

In experiments without calcium, the P_2 fraction was washed once after preincubation in [^{3}H]choline with calcium-free KR containing 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) and resuspended in this modified KR. Incubation experiments were also carried out in this modified KR.

In the experiments where the uptake of choline during preincubation and the release of labeled choline and AcCh were studied, β-bungarotoxin was added at a concentration of 0.1 μM during the preincubation, i.e., while the P_2 fraction was being loaded with [*methyl*-^{3}H]choline, specific activity 0.35 Ci/mmol. A part of this incubated P_2, after washing with eser-

Abbreviations: β-BuTX, β-bungarotoxin; AcCh, acetylcholine; KR, Krebs–Ringer bicarbonate medium.

Table 1. Effect of β-bungarotoxin on AcCh release from synaptosomes

β-Bungarotoxin (M)	AcCh released per mg of protein (cpm ± SEM)		% K^+-stimulated AcCh release $[(b-a)/a] \times 100$
	5.3 mM K^+ (a)	53 mM K^+ (b)	
0	6,399 ± 616	12,986 ± 719	103
10^{-9}	8,433 ± 936	11,998 ± 1,040	42
10^{-8}	10,372 ± 954	12,168 ± 1,247	17
10^{-7}	11,297 ± 804	13,576 ± 1,248	20
10^{-6}	13,589 ± 789	15,236 ± 826	12
10^{-5}	13,851 ± 1,214	15,833 ± 1,155	14
In Ca^{2+}-free medium			
0	7,090 ± 575	8,571 ± 444	21
10^{-9}	7,340 ± 1,155	8,738 ± 1,034	19
10^{-6}	7,239 ± 435	9,014 ± 621	25

ine–KR, was extracted with formic acid/acetone and subjected to electrophoresis, and radioactivity in the AcCh and choline region was measured to get the total label taken up and converted to AcCh. With another part of this incubated P_2, release of AcCh and choline was studied as described earlier.

Kinetics of Choline Uptake. High-affinity choline uptake was determined following the method of Yamamura and Snyder (15) with a slight modification. The P_2 fraction was prepared as described and reconstituted using 5 ml of KR per g of cortex. A portion of this P_2 suspension was kept at 37° for 30 min with or without 0.1 μM β-bungarotoxin, and a 0.1 ml portion from this was added to 1.9 ml of KR containing 0.5 μM choline (specific activity 4 Ci/mmol, also kept at 37°) and the incubation was continued. After various time intervals, the uptake of choline was terminated by removing the tube to ice and adding 5 ml of ice-cold KR containing 4 mM choline and 0.4 mM eserine sulfate; the suspension was centrifuged immediately at 15,000 × *g* for 10 min. The pellet was washed twice with ice-cold 0.9% NaCl–0.2 mM eserine and centrifuged. The accumulated radioactivity was extracted with 2 ml of 1% Triton X-100, 0.2 M NaOH by shaking at 55° for 2–3 hr and then allowing overnight standing at room temperature. The radioactivity of an aliquot was determined by using Aquasol scintillation liquid.

Measurement of Choline Acetyltransferase, Cholinesterase, and Phospholipase A Activity. Choline acetyltransferase (EC 2.3.1.6) activity of the P_2 fraction before and after incubation with β-bungarotoxin was measured essentially according to the method of Roskoski (16).

Acetylcholinesterase (EC 3.1.1.7) was measured following the method of Ellman (17). Phospholipase activity (EC 3.1.1.32) was assayed according to the method of Marinetti (18).

Electron Microscopy. All samples were incubated with or without β-bungarotoxin, as described in *Materials and Methods*, for either 30 or 90 min. Two concentrations of the toxin were used, 10 μM or 0.1 μM. Samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in cacodylate buffer. After dehydration in graded ethanol and propylene oxide, samples were embedded in Vestopal. Silver-gray sections were cut on a Porter-Blum microtome, stained in uranyl acetate and lead citrate, and examined in a Zeiss EM-9A electron microscope.

RESULTS

Effect of β-Bungarotoxin on AcCh Release. As shown in Table 1, the basal release of AcCh, i.e., release in KR containing 5.3 mM KCl, increased linearly as the concentration of β-bungarotoxin was increased from 1 nM to 1 μM and essentially leveled off thereafter. The release of AcCh at high KCl concentrations (53 mM) was virtually unaffected by β-bungarotoxin up to 0.1 μM. At all concentrations of the toxin the difference between low and high K medium, though significant ($P < 0.05$), was considerably less than control. Hence, although the actual amount of labeled AcCh released was increased with rising levels of the toxin, K^+-stimulated release of the transmitter was actually decreased compared to control, as shown in the final column of Table 1. That this effect of β-bungarotoxin required Ca^{2+} regardless of the K^+-concentration of the medium is also shown in the table.

To determine whether the toxin also affected the uptake of choline and its conversion to AcCh, β-bungarotoxin was added during the preincubation together with [^{3}H]choline. Table 2 shows that whereas the level of labeled choline in synaptosomes was independent of β-bungarotoxin, in the presence of β-bungarotoxin radioactive AcCh was only 50% of that present in the control tissue. Again, as can be seen from the table, 39% and 74% of the accumulated AcCh was released from control synaptosomes by 5.3 and 53 mM K^+, respectively, but in the case of toxin-treated preparations, the release was as high as 86% and 97%. Hence, the effect of the toxin on AcCh release was qualitatively similar to the results shown in Table 1, but the actual content of the transmitter was different in treated and control preparations. Little difference was observed in the choline released in control and treated preparations.

Since the diminished accumulation of radioactivity in the P_2 fraction in the presence of the toxin could be due to an effect of β-bungarotoxin on choline acetyltransferase activity, this possibility was then explored.

Table 2. Effect of β-bungarotoxin on synaptosomal uptake of choline and release of AcCh and choline

Incubation conditions	cpm/mg of protein after preincubation		AcCh released per mg of protein (cpm)		Choline released per mg of protein (cpm)	
	AcCh	Ch	5.3 mM K^+	53 mM K^+	5.3 mM K^+	53 mM K^+
Control	12,827 ± 542	8,539 ± 309	4,989 ± 24.7	9,523 ± 88	5,354 ± 123	5,147 ± 166
β-Bungarotoxin (0.1 μM)	6,577 ± 113	7,863 ± 479	5,680 ± 481	6,540 ± 182	4,925 ± 299	5,260 ± 246

Table 3. Effect of β-bungarotoxin on choline acetyltransferase and acetylcholine esterase activity of rat cortical synaptosomes

Enzyme	Specific activity*	
	Control	β-BuTX (0.1 μM)
Choline acetyltransferase	646.4 ± 31	719 ± 29
Acetylcholine esterase	1.1	1.1

* For choline acetyltransferase, pmol of AcCh formed per min/mg of protein; for acetylcholine esterase, μmol of acetylthiocholine hydrolyzed per min/mg of protein.

Effect on Choline Acetyltransferase. Table 3 shows that choline acetyltransferase activity was not significantly affected by β-bungarotoxin; no inhibition was observed regardless of whether the toxin was added directly during the enzyme reaction or when the synaptosomes were preincubated with the toxin for 30 min prior to the enzyme assay. Hence, inactivation of choline acetyltransferase could not be the reason for the reduction in radioactive AcCh in treated synaptosomes. Although it was unlikely as an explanation of the action of β-BuTX, the effect of the toxin on acetylcholinesterase activity was also determined in order to complete our studies on AcCh metabolism. Neither activation nor inhibition of acetylcholinesterase in the synaptosomes was observed. It was, therefore, reasonable to assume that choline uptake might be the process which was affected.

Effect on Choline Uptake. Synaptosomes are capable of selectively accumulating neurotransmitters or their precursors by a sodium-dependent, high-affinity system (15). As shown in Fig. 1, the uptake of choline was negligible at 0° but at 37° synaptosomes accumulated an increasing amount of radioactivity with time. When synaptosomes were incubated with 0.1 μM β-bungarotoxin for 30 min, the uptake of choline was found to be reduced to 27% of control in 3 min. The effect of β-bungarotoxin was the same whether it was preincubated with P_2 for 10, 20, or 30 min, or if β-bungarotoxin was added during the uptake process. Further, this effect of β-bungarotoxin was not reversible by dialysis; inhibition of choline uptake remained the same before and after a 3-hr dialysis in treated as compared to untreated synaptosomes. Hence, it appeared that the diminished accumulation of radioactivity, as shown in Table 2, was not due to any effect of the toxin on the enzyme systems responsible for AcCh synthesis or breakdown but on the high-affinity choline uptake, and it also appeared that the effect of the toxin was irreversible.

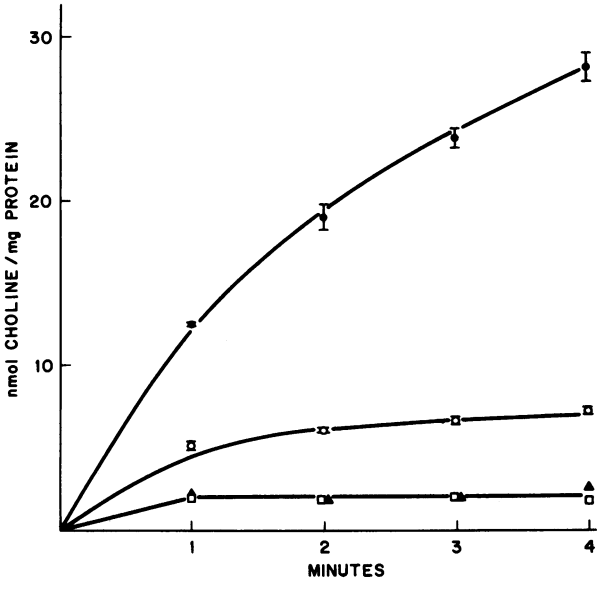

FIG. 1. Effect of β-bungarotoxin on choline uptake by synaptosomes. After a 30-min incubation of the synaptosomes with β-bungarotoxin (0.5 μM), portions were incubated at either 0° or 37° with [^{3}H]choline as described in *Materials and Methods*. ●, control 37°; ▲, control 0°; ○, β-BuTX 37°; □, β-BuTX 0°.

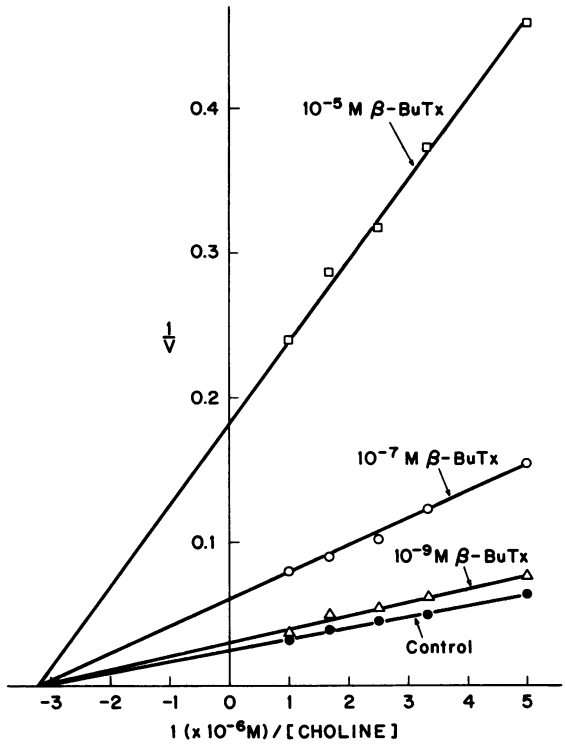

FIG. 2. Lineweaver–Burk plot of velocity of choline uptake versus concentration of choline. Velocity is expressed as nmol of [^{3}H]choline taken up per mg of protein during a 3-min incubation after a 10-min preincubation of synaptosomes with β-BuTX at various concentrations.

In order to obtain further information, a kinetic analysis of choline uptake as affected by the toxin was undertaken. Synaptosomes, treated with various concentrations of β-bungarotoxin, were incubated with concentrations of [^{3}H]choline varying from 0.2 to 1 μM, the specific activity being 4 Ci/mmol in all experiments. As shown in Fig. 2, with increasing concentrations of β-bungarotoxin, choline uptake decreased progressively; from these data the kinetics of uptake were determined by double reciprocal plots to give the K_m and the V_{max}. It is clear from the figure that the toxin, regardless of the concentration, does not affect the K_m (0.23 μM) significantly, but the V_{max} progressively decreased as the concentration of toxin was raised. From these data it was calculated that 50% inhibition of choline uptake resulted from a concentration of toxin of 50 nM.

Effect of Phospholipase A Inhibitor. It has been suggested (9, 19) that endogenous phospholipase A activity is responsible for most of the venom's observed effects on storage and release of neurotransmitters. We therefore used a phospholipase A inhibitor (20) together with β-bungarotoxin to see whether the effects of the toxin would be negated. After determining that an aqueous dispersion of DL-2,3-distearoyloxypropyl(dimethyl)-β-hydroxyethylammonium acetate, at a concentration of 0.3 mg/ml, completely inhibited the phospholipase A activity of the crude venom, we incubated 0.6 mg/ml of this phospholipase A inhibitor with the toxin during

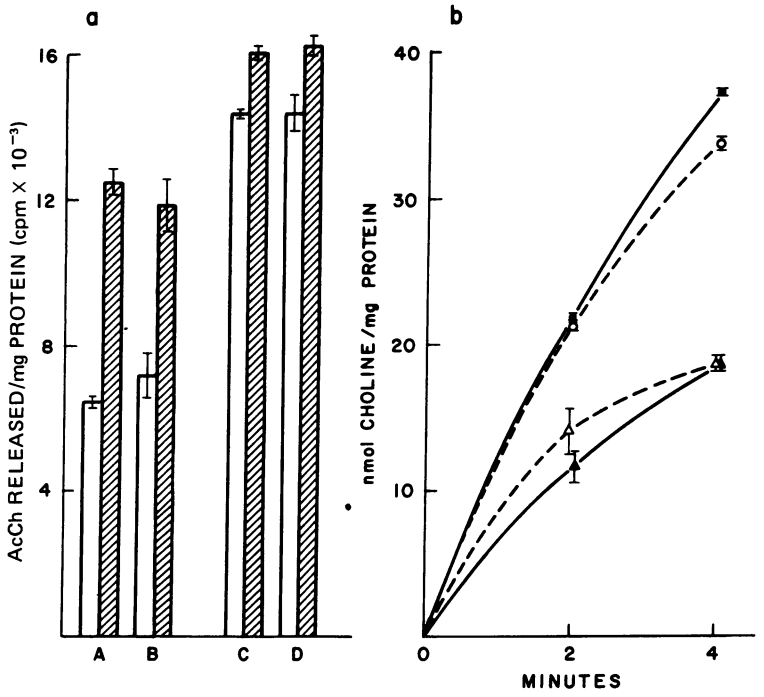

FIG. 3. The effect of the phospholipase A inhibitor. (a) Effect on release of AcCh in the absence and presence of β-BuTX (0.1 μM). Open bars indicate release of transmitter in basal medium (5.3 mM K^+); hatched bars indicate release in a high K^+ (53 mM) medium. A = control synaptosomes, B = control synaptosomes plus phospholipase inhibitor, C = synaptosomes plus β-BuTX, D = synaptosomes plus β-BuTX plus phospholipase inhibitor. Error brackets represent SEM. (b) Effect on choline uptake in the absence and presence of β-BuTX (0.1 μM). ●, choline uptake in control synaptosomes; ○, choline uptake in synaptosomes in presence of phospholipase inhibitor; ▲, uptake in β-BuTX-treated synaptosomes; △, uptake in β-BuTX-treated synaptosomes plus phospholipase inhibitor.

experiments involving release of AcCh or uptake of choline. As shown in Fig. 3, the inhibitor did not significantly affect the release of AcCh from control or toxin-treated synaptosomes either at 5.3 or 53 mM K^+ concentrations. Similarly, the reduced uptake of choline by β-bungarotoxin was also unaffected by the presence of the phospholipase A inhibitor.

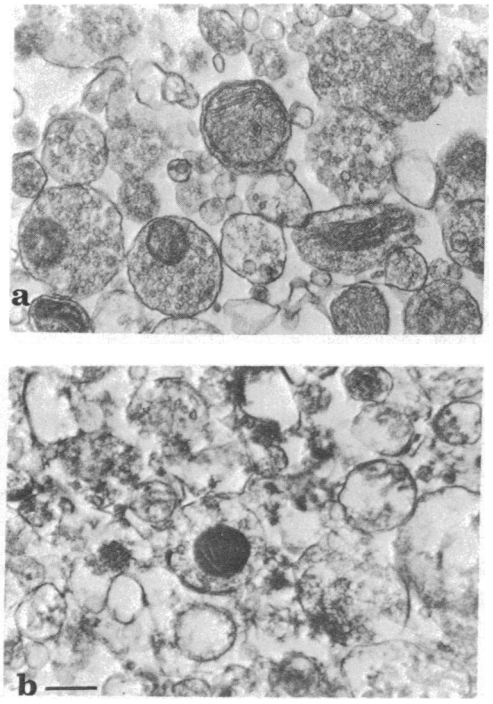

FIG. 4. Electron micrographs of synaptosomes treated with β-BuTX for 20 min at 37°. (a) β-BuTX concentration was 0.1 μM; (b) β-BuTX concentration was 10 μM. The bar = 0.25 μm.

Effect on Ultrastructure. Samples incubated with β-bungarotoxin (0.1 μM) for either 30 or 90 min were morphologically indistinguishable from incubated controls. However, in samples incubated with β-bungarotoxin (10 μM) for 90 min, mitochondria appeared swollen and there was a definite absence of ultrastructure, although synapses and synaptic vesicles seemed less affected (Fig. 4).

DISCUSSION

The electrophysiological studies on the neuromuscular junction (1–5, 7, 26), in which the effects of β-BuTX were first observed, are readily understandable from the neurochemical evidence presented in this paper. We have shown that the initial response of an increase in miniature endplate potentials is due to the rapid release of AcCh by the toxin; the subsequent inhibition of transmission is due to the potent inhibitory effect of β-BuTX on choline transport into the terminal with resultant diminished AcCh content in the tissue. Since recent studies (21) suggest that the high-affinity choline transport system regulates the level of the transmitter in nervous tissue, it is not surprising that synaptic transmission can be blocked by choline transport inhibitors despite the presence of choline-containing phospholipids in the terminal that could serve as a source of the base. As shown in Table 2, the presence of β-BuTX caused a 50% decrease in the AcCh content of the preparation. Chang *et al.* (5) did not find a decrease in the AcCh content of their preparation after prolonged treatment with the toxin. However, these investigators pretreated the phrenic nerve diaphragm preparation with an anticholinesterase. This would result in the accumulation of surplus AcCh, not releasable by nerve stimulation (27, 28), and would thus mask the depletion of the physiologically relevant store of the transmitter. It should be noted that although the choline content was similar in treated and untreated samples (Table 2), in apparent contradiction to our

results on choline transport (Fig. 2), in this experiment both low- and high-affinity transport were measured, whereas only the latter is involved in AcCh synthesis. The curiosity with the β-BuTX-induced release of AcCh is that it is only moderately affected by a depolarizing concentration of K^+ and yet requires Ca^{2+} to effect the release. Although Chang and coworkers did not find a calcium dependency, recent work (26) clearly demonstrated this requirement. This necessity for calcium would tend to support the contention that it is indigenous phospholipase activity (a Ca^{2+}-activated enzyme) in the bungarotoxin that is responsible for the observed effect on release of transmitter. However, we have shown that the addition of a phospholipase inhibitor does not alter the activity of β-BuTX on either release of AcCh or uptake of choline. Of course, we cannot exclude the possibility that the inhibitor assay, which utilizes egg yolk as substrate, might not be sensitive enough to detect the different types of phospholipase activity which may act preferentially on synaptosomal preparations.

Chen and Lee (6) noted that β-BuTX causes mitochondrial swelling, and Kelly and Brown (7) have proposed that the toxin exerts its primary action on mitochondria. In our experiments, at a concentration of β-BuTX of 0.1 μM, mitochondria appear perfectly normal in electron microscopy and cannot be differentiated from control preparations; it is only when the concentration of the toxin is raised to 10 μM that the mitochondrial structure is altered.

Our current understanding of transmitter release mechanism is minimal; we are not even certain of the role of synaptic vesicles in this process (22). Fortunately, we now have a number of agents that act presynaptically and that can be used to clarify this problem. Thus, in addition to β-BuTX, botulinum toxin acts to prevent AcCh release by a mechanism still unknown; black widow spider venom discharges AcCh, possibly by promoting the disruption of synaptic vesicles (23); and collagenase, which prevents release, possibly by interfering with calcium uptake (refs. 13 and 24, and unpublished observations). In addition, organic chemicals such as hemicholinium-3 (25) and juglone (Cooper, unpublished observations) block transmission by interfering with the high-affinity uptake process for choline. It may be assumed that an exploitation of these agents should be profitable in elucidating the mechanisms involved in the release of neurotransmitters.

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