Biochimica et Biophysica Acta, 596 (1980) 81—93

BBA 78609

COMPLETE PURIFICATION OF β-BUNGAROTOXIN

CHARACTERIZATION OF ITS ACTION AND THAT OF TITYUSTOXIN ON SYNAPTOSOMAL ACCUMULATION AND RELEASE OF ACETYLCHOLINE

JOHN W. SPOKES and J. OLIVER DOLLY

Department of Biochemistry, Imperial College, London SW7 2AZ (U.K.)

(Received May 2nd, 1979)

Key words: β-Bungarotoxin purification; Tityustoxin; Synaptosome membrane; Acetylcholine release

Summary

β-Bungarotoxin, a snake venom protein (molecular weight 21 000) that irreversibly blocks release of acetylcholine from nerve terminals, was purified to homogeneity by ion-exchange chromatography and isoelectric focussing. Sodium dodecyl sulphate gel electrophoresis under reducing conditions resolved two subunits of molecular weight 11 400 and 9000. In the presence of deoxycholate, it showed phospholipase activity which was activated by Ca^{2+} but not Sr^{2+}. β-Bungarotoxin and tityustoxin, a polypeptide that prolongs the opening of sodium channels, inhibited choline accumulation by synaptosomes purified from rat cortex. Both toxins also induced release of acetylcholine which was maximal in the presence of Ca^{2+} and showed ED_{50} values of $5 \cdot 10^{-8}$ and 10^{-6} M, respectively *. Unlike tityustoxin, β-bungarotoxin also induced release of choline and cytoplasmic lactate dehydrogenase from synaptosomes, with similar potency, suggesting that it causes some membrane disruption, following its binding to the membrane. The effects of tityustoxin on both accumulation and release were antagonised by tetrodotoxin, which specifically blocks Na^+ channels, indicating that it mediates these effects by depolarization. Thus, these toxins may prove to be useful probes for characterisation of nerve membrane components involved in triggering transmitter release.

Introduction

β-Bungarotoxin and tityustoxin, two of a series of neurotoxins [1] that can affect the release of transmitters [2,3] appear to be useful probes for investi-

* ED_{50} is the concentration of toxin which produces 50% of its maximum measurable effect.

gating components of the release mechanism. Tityustoxin is purified from the venom of the Brazilian yellow scorpion *Tityus serrulatus* [4]; it increases the average time for which potential-dependent Na^+ channels are open, thereby causing depolarization of the membrane [5,6]. This action is also shown by toxins from other scorpion venoms [7—10] and is antagonised by tetrodotoxin, an alkaloid which specifically and reversibly blocks the ion filter of the Na^+ channel [11].

β-Bungarotoxin [12] and another presynaptic toxin from the venom of *Bungarus multicinctus*, produce a triphasic effect on the spontaneous [13,14] and evoked [13,15] release of neurotransmitters in nerve-muscle preparations. An initial decrease in release is followed by a transient increase and, eventually, complete blockade. Purified preparations of β-bungarotoxin show a phospholipase A_2 activity in the presence of deoxycholate [15,16]. The importance of this activity was examined using enzymically inactive toxins [13—15] or those in which the phospholipase activity was inhibited by removal of Ca^{2+} [16—18] and its replacement by Sr^{2+}; the latter can substitute in the process of transmitter release [19]. In the absence of enzyme activity the toxicity is greatly reduced and only the first phase of the toxin's action at the neuromuscular junction is observed [13,15,17]. Comparison of the toxicity of β-bungarotoxin with those of snake venom phospholipases shows that it cannot be due solely to the enzyme activity [16]. It has been suggested that the first phase of the action of β-bungarotoxin is due to its binding to a component of the release mechanism [17]; this could account for its specificity for neuronal membranes.

We report a new method for preparing homogeneous β-bungarotoxin and examine its actions, together with those of tityustoxin, on the accumulation and release by synaptosomes of choline and acetylcholine, respectively. Similar studies, using the putative neurotransmitters glutamate and γ-aminobutyric acid, have been carried out in this laboratory [2,20].

Materials and Methods

B. multicinctus venom was obtained from Miami Serpentarium Laboratories, U.S.A. [*methyl*-^{3}H]Choline chloride (specific radioactivity 13 Ci/mmol) was supplied by the Radiochemical Centre, Amersham, U.K. Soluene, Bio-lyte (pH 8—10) and Ampholine (pH 7—9 and 9—11) carrier ampholytes and egg yolk phosphatidylcholine (grade 1) were purchased from Packard, Bio-Rad Laboratories, L.K.B. and Lipid Products, Surrey, U.K., respectively. Sodium deoxycholate, bee venom phospholipase A_2, α-chymotrypsinogen A, cytochrome *c*, ovalbumin, myoglobin, tetrodotoxin and veratrine were from Sigma. Tityustoxin, purified from the venom of the Brazilian yellow scorpion *T. serrulatus*, and characterised as previously described [4], was kindly provided by Dr. C. Diniz. Its LD_{50} measured in mice was 0.1 μg/g body weight. All other chemicals used were analytical grade.

Fractionation of B. multicinctus venom. Chromatography on CM-Sephadex was performed by a modification of the procedure used by Lee et al. [12].

Preparative isoelectric focussing. Final purification of β-bungarotoxin was carried out by isoelectric focussing in a flat bed of Sephadex G-75 using an LKB 2117 Multiphor apparatus as described by Winter et al. [21]. Focussing was performed in a gel containing 1% (w/v) Bio-lyte (pH 8—10) at 8 W constant

power for 16 h or, preferably, with 2% (w/v) Pharmalyte (pH 8—10.5) ran at 10 W constant power for 4.5 h, after preforming the gradient at 8 W for 45 min. A print of the bed was prepared and stained for protein. The major band was eluted from the resin and the ampholytes removed by dialysis.

Analytical isoelectric focussing. This was carried out for 4 h at 3 W constant power in a horizontal, water-cooled, slab of polyacrylamide (5.4% (w/v) acrylamide, 0.17% (w/v) bisacrylamide) which contained 9.8% (w/v) sucrose and 1.1% (w/v) of both pH 7—9 and 9—11 ampholytes. Electrical contact was made through filter paper wicks soaked in 1% (w/v) pH 9—11 ampholyte solution at the cathode and 0.1% (w/v) pH 7—9 ampholyte solution at the anode. Protein bands were visualised by precipitation with trichloroacetic acid.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis was carried out at pH 4.5 in a β-alanine/acetate disc system as described by Reisfeld et al. [22] and protein bands were stained with Coomassie G-250. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out by the method of Swank and Munkres [23] using a gel containing 12.5% (w/v) acrylamide, 1.25% bisacrylamide and 8 M urea; samples were run under non-reducing and reducing (10% (v/v) β-mercaptoethanol in sample buffer) conditions. After fixing in 40% trichloroacetic acid the gel was stained with Coomassie R-250 as described by Swank and Munkres [23]. Ovalbumin, α-chymotrypsinogen, myoglobin, cytochrome *c* and CNBr-cleaved fragments of the latter were used as molecular weight markers.

Toxicity and phospholipase assays. Whole animal toxicity was determined by intraperitoneal injection of toxin (dissolved in 0.8% NaCl containing 0.5 mg bovine serum albumin/ml) into 25—30-g mice. The ability of the toxin to block neuromuscular transmission in a frog sartorius nerve-muscle preparation [15] was also tested.

Phospholipase activity was assayed using a Radiometer autotitrator as a pH stat [15]. Activities were calculated from the initial slope and expressed as μmol of H^+ liberated · min^{-1} · mg^{-1} of protein.

Measurement of choline accumulation by synaptosomes. Synaptosomes were prepared from rat cerebral cortex, by a modification of the method of Gray and Whittaker [24], as described by de Belleroche and Bradford [25]. The synaptosome fraction from the sucrose density gradient was diluted with an equal volume of cold Krebs/phosphate medium (20 mM Na_2HPO_4, 1.25 mM KH_2PO_4, 125 mM NaCl, 5 mM KCl, 0.74 mM $CaCl_2$, 1.3 mM $MgSO_4$ and 10 mM glucose, saturated with 95% O_2/5% CO_2, adjusted to pH 7.4) instead of deionised water. Measurement of rates of lactate production and O_2 uptake indicated the viability of this preparation [2,20]. The accumulation of [^{3}H]-choline by synaptosomes was measured using a filtration assay [26]. [^{3}H]-Choline (final concentration 0.5 μM; specific radioactivity 4 Ci/mmol) was added to a synaptosome suspension (2—3 mg of protein/ml) in Krebs/phosphate medium. Samples of this suspension were then added to tubes containing small volumes of toxin solution or water. At various times, aliquots (100 μl) of these suspensions were removed following brief mixing, and placed under suction onto glass fibre filters (Whatman GF/C). The filters were then washed twice with 2.5 ml Krebs/phosphate medium, dried in air and counted in a toluene-based scintillant containing 10% (v/v) Soluene [20]. Controls, containing

osmotically lysed synaptosomes were incubated at 4°C and otherwise treated similarly.

Measurement of acetylcholine and choline release from synaptosomes. Synaptosomes for experiments on release were preloaded by incubation with [^{3}H]choline (1.7 μM; specific radioactivity 1 Ci/mmol) for 20 min at 37°C in a modified Krebs/phosphate medium containing 1.8 mM KCl and 128.2 mM NaCl. The synaptosomes were washed twice by centrifugation at 27 000 × *g* for 2 min and resuspension in modified Krebs/phosphate medium; final resuspension was in the same medium containing 10 mM eserine sulphate. For experiments in the absence of calcium, the synaptosomes were preloaded using medium in which calcium was replaced iso-osmotically by sucrose; subsequent washing and final resuspension were in calcium-free medium containing either the equivalent concentration of $SrCl_2$ or 5 mM EGTA. Aliquots of the bulk pre-loaded suspension were then transferred to tubes containing the requisite amounts of toxin solutions and/or KCl and/or the equivalent volume of water; KCl was added to a final concentration of 23 mM. In experiments involving tetrodotoxin, the toxin solution or the same volume of water was added to the bulk synaptosome suspension and preincubated for 5 min before aliquots were transferred to incubation tubes.

The incubation was terminated after 20 min at 25°C by centrifugation of the tubes at 10 000 × *g* for 5 min. Supernatants were diluted with equal volumes of formic acid/acetone (1 : 3, v/v) and recentrifuged. Aliquots of the latter supernatants were subjected to high-voltage paper electrophoresis to separate choline and acetylcholine [27]. The spots were stained with I_2 vapour, cut out and their radioactivity determined by liquid scintillation counting as noted above. Two sets of control incubations were performed; one was terminated at 0 min while the second was incubated for 20 min; the former was subtracted from the values obtained for all other samples.

Other determinations. Lactate dehydrogenase activity was assayed as described by Bergmeyer [28]. Acetylcholinesterase activity was measured by the method of Ellman et al. [29]. Synaptosome pellets were digested in 0.31 M NaOH and protein was assayed by the method of Lowry et al. [30].

Results

Purification of β-bungarotoxin

Chromatography of *B. multicinctus* venom on CM-Sephadex C-50 gave an elution profile (Fig. 1) similar to that obtained by Lee et al. [12]. Peak V, which contains the material termed β-bungarotoxin by these workers was pooled, desalted on a Sephadex G-25 column and freeze-dried. Polyacrylamide gel electrophoresis of this toxin under native conditions showed two protein bands (Fig. 2, upper); analytical isoelectric focussing revealed at least three contaminants. We were unable to prepare homogeneous toxin by a further series of CM-cellulose [16] and SP-Sephadex [31] ion-exchange columns or by gel filtration on Sephadex G-75. However, preparative isoelectric focussing over a narrow pH range resolved four minor bands from the major component (Fig. 3). The major band, when eluted and dialysed to remove ampholytes, showed only one band on native polyacrylamide gel electrophoresis (Fig. 2, lower) and ana-

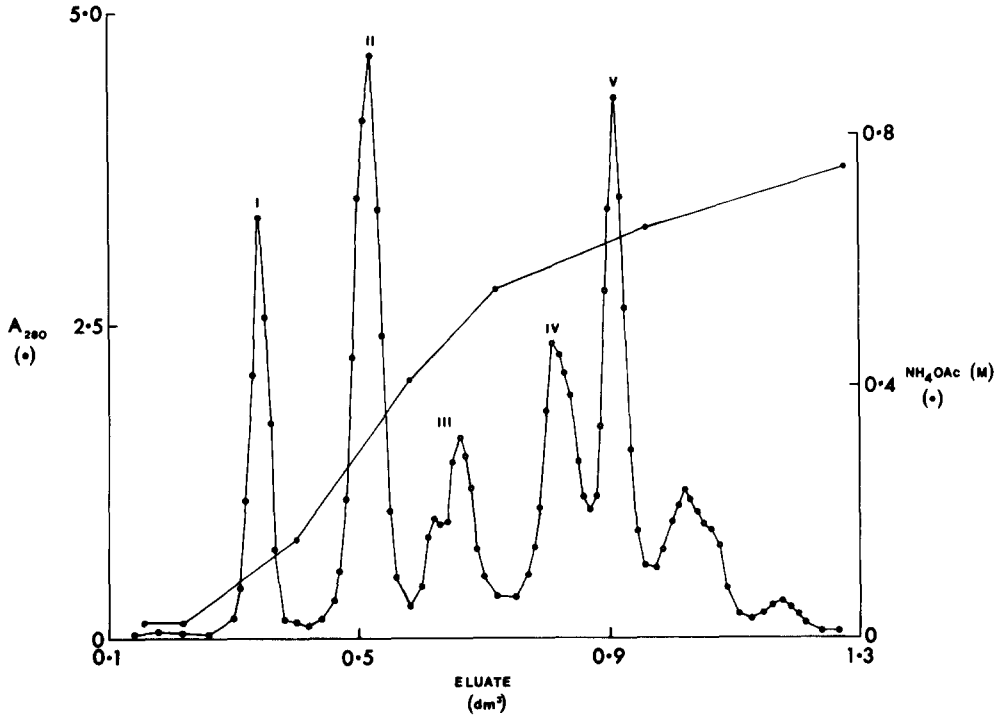

Fig. 1. Fractionation of *B. multicinctus* venom by ion-exchange chromatography. 1 g of venom was applied to a CM-Sephadex C-50 column (2.4 × 67 cm), equilibrated with 0.05 M ammonium acetate, pH 7.0, and was eluted with a convex gradient using 400 ml of the latter and 0.9 M ammonium acetate, pH 7.4; 4-ml fractions were collected. Protein was determined by A_{280} (○) and ammonium acetate concentration (•) was calculated from conductivity measurements. Peak V (0.87—0.95 dm^3) was pooled.

lytical isoelectric focussing (Fig. 3); it will be referred to as β-bungarotoxin. This toxin also gave a single protein band on sodium dodecyl sulphate polyacrylamide gel electrophoresis, under non-reducing conditions, with a molecular weight of 16 800 ± 800 (n = 3); it is, therefore, homogeneous with respect to charge and size.

Under native conditions a molecular weight of 21 000 was found for the toxin by gel filtration on Sephadex G-75. After reduction two protein bands with molecular weights of 9000 ± 300 (n = 3) and 11 400 ± 250 (n = 3) were separated by sodium dodecyl sulphate electrophoresis. These latter figures are in general agreement with those reported [15,32—34] and were obtained using a more comprehensive range of standards than were those which we previously reported [2]. The LD_{50} in mice was less than 0.01 μg/g body weight; at concentrations of 0.5, 2.75 and 5.0 μg/ml, a complete blockade of neurotransmission was produced after 6.9, 4.3 and 1.7 h, respectively, in a frog sartorious nerve-muscle preparation, as previously reported [15,16,35].

Phospholipase assays on β-bungarotoxin

Negligible activity was found in the absence of deoxycholate (Table I) as we had previously observed using a conductimetric assay [2]. In contrast, when

Fig. 2. Polyacrylamide gel electrophoresis of β-bungarotoxin. The resolving gel contained 15% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide. Samples were applied at the right (anodic) end of the gel and electrophoresis was carried out at pH 4.5 in a β-alanine/acetate buffer system using 25 μg of toxin; protein was stained with 0.04% (w/v) Coomassie G-250 in 3.5% (w/v) perchloric acid. Upper: Peak V from CM-Sephadex column (Fig. 1). Lower: Pure β-bungarotoxin obtained from preparative isoelectric focussing of peak V material.

the detergent was added appreciable phospholipase activity was observed [15, 16,18] but its specific activity was much lower than that of purified bee venom phospholipase A_2. In the presence of deoxycholate, the phospholipase activity of the toxin was greatly reduced when Sr^{2+} was substituted for Ca^{2+}; moreover,

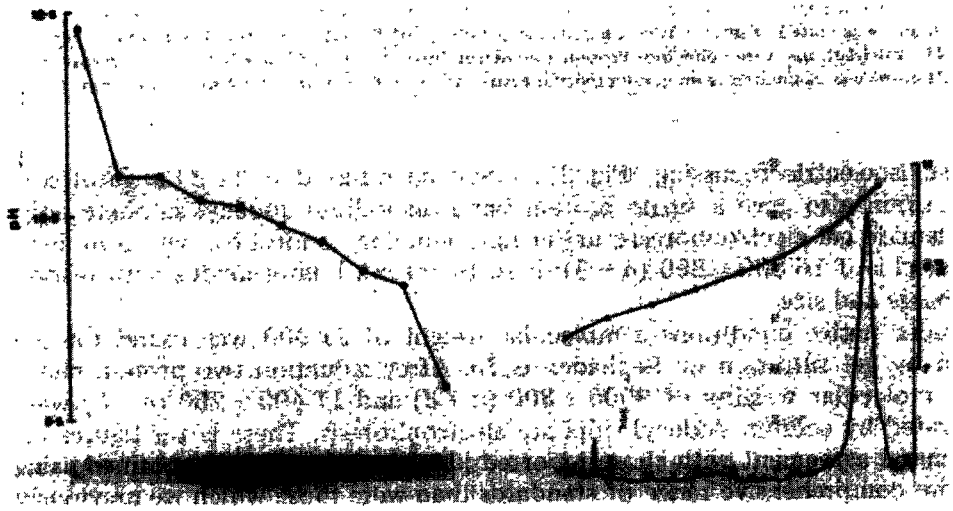

Fig. 3. Isoelectric focussing of β-bungarotoxin. Left: Preparative isoelectric focussing of peak V (20 mg) from the CM-Sephadex column (Fig. 1) was performed in a flat bed of Sephadex G-75. After focussing the pH gradient (•) was measured with a microelectrode and a paper print of the bed was fixed in 10% trichloroacetic acid and stained with 0.2% Coomassie R-250 in methanol/water/acetic acid (5 : 5 : 1, by vol.) and destained in the same solvent mixture. Right: Isoelectric focussing in a polyacrylamide gel using pH 7—9 and 9—11 ampholytes, of a toxin sample (60 μg) purified by preparative isoelectric focussing (as above). After focussing, the pH gradient (•) was measured and the protein in the gel was precipitated with 40% trichloroacetic acid, washed in 10% trichloroacetic acid to remove the ampholytes and scanned at 450 nm.

TABLE I

CHARACTERISTICS OF PHOSPHOLIPASE ACTIVITIES OF β-BUNGAROTOXIN AND BEE VENOM PHOSPHOLIPASE A_2

Assays were carried out at 37°C using a pH stat. The reaction mixture (volume 5.5 ml) contained 10 μmol of dispersed egg yolk phosphatidylcholine and 100 mM NaCl. Additions of 10 mM $CaCl_2$, 10 mM $SrCl_2$, 0.1 mM EGTA and 0.18 mM sodium deoxycholate were made as indicated.

	Phospholipase activity (μequiv. · min^{-1} · mg^{-1} of protein)	
	β-Bungarotoxin	Bee venom phospholipase A_2
$CaCl_2$	0.6	326
$CaCl_2$ + deoxycholate	63	1345
$SrCl_2$ + deoxycholate	2.6	—
EGTA + deoxycholate	0	0

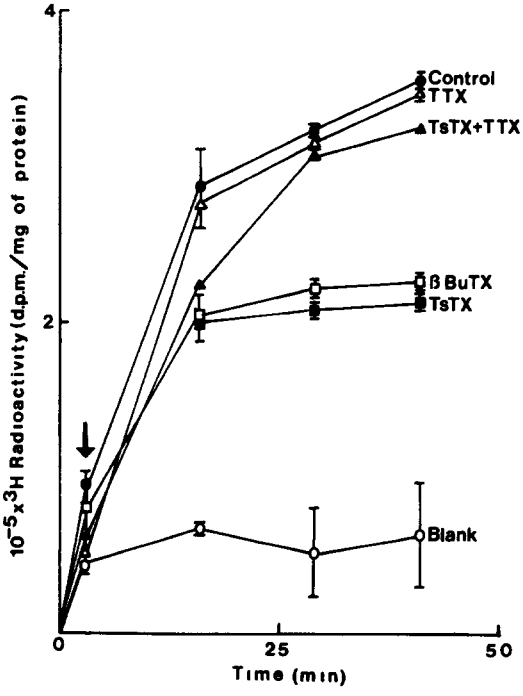

Fig. 4. Effect of β-bungarotoxin and tityustoxin on high-affinity uptake of [^{3}H]choline by synaptosomes. The time course of accumulation of radioactivity (±S.D., $n = 2$) by synaptosomes at 25°C in Krebs/phosphate medium containing [^{3}H]choline (0.5 μM; 4 Ci/mmol) was measured by a filtration assay as described in Materials and Methods. Control incubations (●) contained no toxin additions and were incubated at 25°C. Blanks (○) were incubated at 4°C and contained synaptosomes which had been osmotically lysed in cold deionised water. Other samples, incubated at 25°C, contained $2.3 \cdot 10^{-7}$ M β-bungarotoxin (□); $3 \cdot 10^{-6}$ M tityustoxin (■); $1.5 \cdot 10^{-5}$ M tetrodotoxin (△), and $3 \cdot 10^{-6}$ M tityustoxin + $1.5 \cdot 10^{-5}$ M tetrodotoxin (▲). Tetrodotoxin was added at zero time; β-bungarotoxin and tityustoxin were added at the time indicated by the arrow.

it was completely abolished in the absence of Ca^{2+} and the presence of 0.1 mM EGTA.

Effect of β-bungarotoxin and tityustoxin on choline accumulation by synaptosomes

Under the conditions used in these experiments the synaptosomes showed high-affinity uptake of radioactive choline (Fig. 4) [36]. Nonspecific accumulation of radioactivity, as shown by the blank, was relatively low. Both β-bungarotoxin and tityustoxin decreased the net accumulation of radioactive choline by synaptosomes; this action of tityustoxin was antagonised by tetrodotoxin which, on its own, had little effect on uptake (Fig. 4). It is interesting to note that addition of β-bungarotoxin and tityustoxin, 2—3 min after resuspension of the synaptosomes in medium containing radioactive choline, did not produce an effect on accumulation earlier than 15 min. However, in a separate experiment addition of either toxin at 15 min was shown to produce an immediate inhibition of accumulation of radioactivity. Under the conditions used in these uptake experiments, neither of the toxins caused a net decrease in the radioactive content of the synaptosomes, indicating that their inhibition of accumulation was not due to gross lysis of the synaptosomes.

Effect of β-bungarotoxin and tityustoxin on the release of acetylcholine and choline from synaptosomes

Synaptosomes, preloaded with [^{3}H]choline, showed an increased release of [^{3}H]acetylcholine when the K^+ concentration was raised from 3 to 23 mM (Table II); K^+-evoked release was dependent upon the presence of Ca^{2+} (Table II, series a). The amount of choline released under the same conditions was much smaller and was not Ca^{2+} dependent. Enzymic hydrolysis of acetylcholine did not contribute to the amount of choline in the supernatant as cholinesterase activity was undetectable in the presence of eserine sulphate. Veratrine also stimulated acetylcholine release; at a concentration of 25 μg/ml it gave a 6—7-fold increase (in 20 min) over the amount released by 3 mM K^+ and its action was completely inhibited by $5 \cdot 10^{-6}$ M tetrodotoxin (data not shown). Therefore, this synaptosome preparation exhibits many important characteristics of the physiological release process. β-Bungarotoxin stimulated release of acetylcholine from pre-loaded synaptosomes (Table II, series b and c), exhibiting an ED_{50} of $5 \cdot 10^{-8}$ M (Fig. 5a). The dose-response curve for choline release showed the same ED_{50} but with a smaller increase. β-Bungarotoxin also produced an increase in acetylcholine release in medium containing 23 mM K^+; this increase was usually less than the sum of those contributed by K^+ and toxin alone (Table II, b and c). Since β-bungarotoxin possesses phospholipase activity, the possibility of lysis of synaptosomes being responsible for the release of previously accumulated transmitter was investigated by measuring the release of lactate dehydrogenase, a cytoplasmic marker (Fig. 5a). The dose-response curve for the effect of β-bungarotoxin on lactate dehydrogenase release was very similar to that for its stimulation of acetylcholine and choline release. When Ca^{2+} was replaced by Sr^{2+} the increase in acetylcholine and choline release induced by β-bungarotoxin was inhibited although that caused by depolarisation with 23 mM K^+ was enhanced (Table II, series b). Tityustoxin

TABLE II

THE EFFECTS OF β-BUNGAROTOXIN AND TITYUSTOXIN ON THE RELEASE OF ACETYLCHOLINE AND CHOLINE FROM SYNAPTOSOMES

Synaptosomes which had been preloaded with [^{3}H]choline were incubated at 25°C for 20 min and the amounts of [^{3}H]acetylcholine and [^{3}H]choline released into the supernatant were determined as described in Materials and Methods. The values are the mean (± S.D.) of those obtained from two separate incubations using a single synaptosome preparation. Incubations contained, as noted, β-bungarotoxin, tityustoxin or an increased K^+ concentration of 23 mM. Three separate series of experiments were carried out in which incubations were performed in modified Krebs/phosphate medium and in similar medium with the following changes. Series a: Ca^{2+} omitted and 5 mM EGTA added. Series b: Ca^{2+} replaced isosmotically by Sr^{2+}. Series c: samples preincubated with $5 \cdot 10^{-6}$ M tetrodotoxin. Data are in dpm released/mg of protein.

	Acetylcholine	Choline	Acetylcholine	Choline
			—Ca^{2+}/+EGTA	
Series a				
Control	86 ± 46	22 ± 12	70 ± 124	1 ± 25
23 mM K^+ + tityustoxin (10^{-5} M)	1039	272	666 ± 8	326 ± 6
23 mM K^+	293 ± 104	60 ± 95	113 ± 14	83 ± 20
Tityustoxin (10^{-5} M)	672 ± 139	171 ± 32	568 ± 113	132 ± 60
			—Ca^{2+}/+Sr^{2+}	
Series b				
Control	417 ± 61	61 ± 4	253 ± 16	39 ± 38
β-Bungarotoxin (10^{-7} M)	882 ± 67	372 ± 33	473 ± 54	128 ± 58
23 mM K^+	595 ± 142	90 ± 6	776 ± 128	162 ± 34
23 mM K^+ + β-Bungarotoxin (10^{-7} M)	1105 ± 90	395 ± 73	851 ± 161	222 ± 52
			+ Tetrodotoxin	
Series c				
Control	200 ± 147	7 ± 29	94 ± 47	73 ± 3
Tityustoxin (10^{-6} M)	663 ± 17	151 ± 34	184 ± 72	167 ± 21
β-Bungarotoxin ($5 \cdot 10^{-8}$ M)	693 ± 1	276 ± 11		
Tityustoxin (10^{-6} M) + β-bungarotoxin ($5 \cdot 10^{-8}$ M)	731 ± 55	226 ± 5		
23 mM K^+	504 ± 110	143 ± 28	610 ± 50	67
23 mM K^+ + tityustoxin (10^{-6} M)	580 ± 80	164 ± 52	576 ± 75	156 ± 28
23 mM K^+ + β-bungatotoxin ($5 \cdot 10^{-8}$ M)	771 ± 84	263 ± 125		
23 mM K^+ + tityustoxin (10^{-6} M) + β-bungarotoxin ($5 \cdot 10^{-8}$ M)	777 ± 47	196 ± 25		

also produced an increase in acetylcholine release from synaptosomes (Table II, series a and c); its ED_{50} was approx. 10^{-6} M (Fig. 5b) and it is therefore considerably less potent than β-bungarotoxin (Fig. 5a). Tityustoxin, unlike β-bungarotoxin, had much less effect on choline than on acetylcholine release and furthermore, the dose-response curve appears to be shifted to higher concentrations. In direct contrast to β-bungarotoxin, it released negligible lactate dehydrogenase activity except at very high concentrations (Fig. 5b). Tityustoxin produced an increase in acetylcholine release in the presence of 23 mM K^+ (Table II, series a and c); the increase due to 23 mM K^+ and tityustoxin was less than that due to tityustoxin alone (Table II, series a and c). The ability of tityustoxin to induce release of acetylcholine, but not choline, was maximal in the presence of Ca^{2+} (Table II, series a). Likewise, the effects of tityustoxin on release of acetylcholine only were inhibited by tetrodotoxin (Table II, series c).

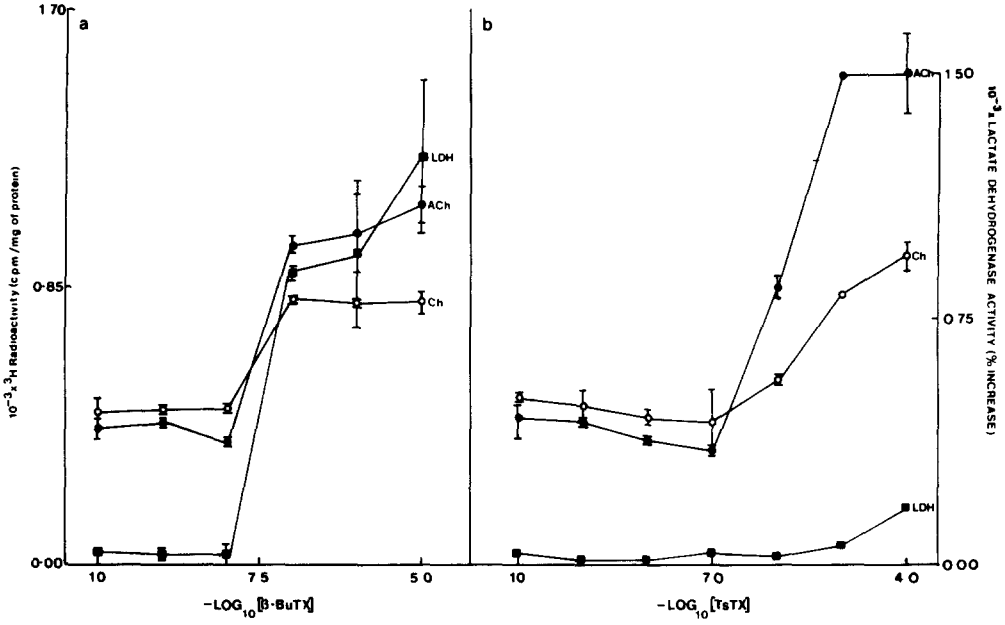

Fig. 5. The concentration dependence of β-bungarotoxin and tityustoxin-stimulated release, from synaptosomes, of acetylcholine, choline and lactate dehydrogenase. The amounts of [^{3}H]acetylcholine (●) and [^{3}H]choline (○) released after incubation of pre-loaded synaptosomes for 20 min at each toxin concentration were determined as described in Materials and Methods. Released lactate dehydrogenase activity (■), measured as described in Materials and Methods, is a percentage increase in the amount present in the supernatant from control samples incubated without toxin addition. The values shown are the means (±S.D.) of those obtained from two separate incubations using a single synaptosome preparation. It was previously shown that release of both acetylcholine and choline was linear with time, for 40 min, at the highest toxin concentrations used.

The increases in transmitter release caused by tityustoxin and β-bungarotoxin, in either 3 or 23 mM K^+, were not additive (Table II, series c). This was not due to a maximal rate of release being reached since in the same experiment, veratrine (at a concentration of 25 μg/ml) released almost twice as much acetylcholine over the same incubation period.

Discussion

Contrary to published observations [15,16,31,33,34,37] β-bungarotoxin prepared from the venom by ion-exchange chromatography could not be further purified by gel filtration or additional ion-exchange steps; this may reflect differences in the venom samples or the elution conditions used. However, isoelectric focussing in a narrow range pH gradient separated four contaminants (Fig. 3); the resulting toxin was homogeneous on narrow range analytical isoelectric focussing (Fig. 3), an essential criterion of purity. It is possible that such contaminants were not previously detected in purified preparations of β-bungarotoxin when isoelectric focussing was not used [15,31,37] or steeper

pH gradients were employed [16,33,34].

It should be also noted that the amino acid compositions of four of these preparations are similar [15,32,33,34], so that the species we have separated may represent 'isotoxins' similar in composition to the major form. The molecular weight of the toxin (21 000) obtained by gel filtration [32] is identical to that calculated from the amino acid sequence [38] and also agrees with the sizes of its subunits measured by urea-sodium dodecyl sulphate electrophoresis [15,32—34]. However, for unknown reasons, a somewhat low value was observed for unreduced toxin with the latter technique; interestingly a molecular weight of 18 500 was obtained by equilibrium sedimentation [32].

It has now been shown that β-bungarotoxin stimulates the release, from preloaded brain synaptosomes, of radioactive acetylcholine (Table II) [27], glutamate [2,20], γ-aminobutyrate [2,20,26] and noradrenalin [26]. This is probably analogous to the increased release during the second phase of the toxin's action on the intact neuromuscular junction. Moreover, as in the latter case, it may precede the eventual toxin blockade of excitatory (glutamate and/or aspartate) transmission observed in slices of rat olfactory cortex and dentate gyrus and also at cholinergic synapses in sympathetic ganglion (Halliwell, J., Marsh, S., Brown, D. and Dolly, J.O., unpublished results). The increase caused by tityustoxin in the release of transmitters from synaptosomes (Table II) [2,20] agrees with its observed action on neurotransmission in intact synapses [6,39—41]. The availability of tetrodotoxin, has enabled the mode of action of tityustoxin to be much more easily characterised than that of β-bungarotoxin for which no such antagonist is yet available.

β-Bungarotoxin [2,20,26,27,42] and tityustoxin [2,20] both inhibit the net accumulation of amino acid transmitters or choline by synaptosomes (Fig. 4). It is necessary to establish if these effects represent direct inhibition of the high-affinity uptake process or increased release of already accumulated transmitter. Furthermore, depolarization of the membrane may contribute to these effects since it has been reported that uptake of glutamate into brain synaptosomes is dependent on membrane potential [43]. Tetrodotoxin has no effect on the net accumulation of transmitter [20] or choline by synaptosomes (Fig. 4), nor on the binding to the membrane of a scorpion toxin similar to tityustoxin [44], but does inhibit tityustoxin-induced depolarization of the membrane. Thus, if the effect of tityustoxin on transmitter uptake is a direct one due to its binding at a site other than the action potential-sodium channel complex, its decrease of transmitter accumulation should not be affected by tetrodotoxin. In fact, this was not the case for choline (Fig. 4), γ-aminobutyrate, or glutamate [20]. It may reasonably be concluded, therefore, that the effects of tityustoxin are due solely to membrane depolarization. Although effects of β-bungarotoxin on accumulation and release of transmitters cannot be studied separately, some interesting points can be made regarding its precise action. The effect of β-bungarotoxin on transmitter release does not involve Na^+ or K^+ channels since it is not antagonised by either tetrodotoxin [37,45, 46] or tetraethylammonium ions [20]. If the mechanism by which β-bungarotoxin acts does not involve membrane depolarization then its effects might be expected to be additive with those of tityustoxin and elevated external K^+ concentration. This is not the case for release of acetylcholine (Table II),

γ-aminobutyrate or glutamate [20]. Following early addition of either β-bungarotoxin or tityustoxin to synaptosomes freshly suspended in medium containing a high concentration of radiolabelled choline, a period of several minutes is required before any effect on the time course of uptake is seen (Fig. 4). This suggests that some labelled choline must be taken up before the apparent decrease in the rate of accumulation is observed and indicates the toxins affect, primarily, transmitter release.

β-Bungarotoxin possesses a phospholipase activity which is important for its action. This is shown by the fact that Sr^{2+} cannot substitute for Ca^{2+} in supporting the toxin's effect on the release from synaptosomes of acetylcholine (Table II), glutamate or γ-aminobutyrate [20] and at the neuromuscular junction [16—18]. Unlike tityustoxin, β-bungarotoxin releases choline and the cytoplasmic marker lactate dehydrogenase in addition to the transmitter acetylcholine; the dose-response curves for all three being very similar (Fig. 5). This strongly suggests that the mechanisms by which β-bungarotoxin increases release of the transmitter substance and the marker enzyme are related and, in view of the phospholipase activity, that it involves some disruption of the membrane. The action of β-bungarotoxin does not result in gross lysis of the nerve terminal as the maximum amount of lactate dehydrogenase activity released is only a fraction of that liberated by 2% Triton X-100. Also in uptake experiments (Fig. 4), treatment with toxin does not lead to a net decrease in accumulated radioactivity and ultrastructural damage is only observed in the electron microscope at very high toxin concentrations [27].

It appears likely that the first stage of β-bungarotoxin action, a transient decrease in transmitter release, is due to the initial binding of the toxin [17] to a protein component of the presynaptic plasma membrane [47], probably through the B subunit. The second and third phases are due to the phospholipase activity of the A subunit, possibly at the phase boundary between stationary, annular lipid and the surrounding fluid bilayer [48]. The depolarisation of the nerve terminal resulting from disruption of the membrane would produce an increased rate of release of transmitter and a decreased net rate of re-uptake or accumulation of precursor [45]. The effects of β-bungarotoxin observed using synaptosome preparations, or intracellular recording at intact synapses, are a consequence of the initial binding of the toxin and are probably an indirect measure of this binding. Eventual blockade of neurotransmission may result from energy depletion due to uncoupling of mitochondria by free fatty acids [37,49], localised ultrastructural damage [27], or a latent effect resulting from the initial toxin binding to transmitter release sites [13,17,50] or Ca^{2+} channels [13].

Acknowledgements

We thank C.R. Diniz for providing purified tityustoxin and C.K. Tse for help with some of the experiments. This work was supported by the M.R.C. as part of a programme grant and by a studentship (to J.W.S.).

References

1 Thesleff, S. (1977) Naunyn-Schmiedeberg's Arch. Pharmacol. 297, S5—S7
2 Dolly, J.O., Tse, C.K., Spokes, J.W. and Diniz, C.R. (1978) Biochem. Soc. Trans. 6, 652—654

3 Freire-Maia, L., Cunha-Melo, J.R., Gomez, M.V., Tafuri, W.L., Maria, T.A., Calixto, S.L. and Futuro-Neto, H.A. (1976) in Animal, Plant and Microbial Toxins (Ohsaka, A., Hayashi, K. and Sawai, Y., eds.), Vol. 2, pp. 273—285, Plenum Publishing, New York
4 Gomez, M.V. and Diniz, C.R. (1966) Mems. Inst. Butantan 33, 899—902
5 Linden, C.D. and Raftery, M.A. (1976) Biochem. Biophys. Res. Commun. 72, 646—653
6 Warnick, J.E., Albuquerque, E.X. and Diniz, C.R. (1976) J. Pharmacol. Exp. Ther. 198, 155—167
7 Bernard, P., Couraud, F. and Lissitzky, S. (1977) Biochem. Biophys. Res. Commun. 77, 782—788
8 Catterall, W.A. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1782—1786
9 Romey, G., Abita, J.P., Chichereportiche, R., Rochat, H. and Lazdunski, M. (1976) Biochim. Biophys. Acta 448, 607—619
10 Tazieff-Depierre, F. (1975) C.R. Acad. Sci. Paris, Ser. D. 280, 1181—1184
11 Henderson, R., Ritchie, J.M. and Strichartz, G.R. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3936—3940
12 Lee, C.Y., Chang, S.L., Kau, S.T. and Luh, S.H. (1972) J. Chromatogr. 72, 71—82
13 Livengood, D.R., Manalis, R.S., Donlon, M.A., Masukawa, L.M., Tobias, G.S. and Shain, W. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1029—1033
14 Abe, T., Limbrick, A.R. and Miledi, R. (1976) Proc. R. Soc. Lond. B. 194, 545—553
15 Abe, T., Alema, S. and Miledi, R. (1977) Eur. J. Biochem. 80, 1—12
16 Strong, P.N., Goerke, J., Oberg, S.G. and Kelly, R.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 178—182
17 Abe, T. and Miledi, R. (1978) Proc. R. Soc. Lond. B. 200, 225—230
18 Chang, C.C., Su, M.J., Lee, J.D. and Eaker, D. (1977) Naunyn-Schmiedeberg's Arch. Pharmacol. 299, 155—161
19 Dodge, F.A., Jr., Miledi, R. and Rahamimoff, R. (1969) J. Physiol. 200, 267—283
20 Tse, C.K., Dolly, J.O. and Diniz, C.R. (1980) Neuroscience 5, 135—143
21 Winter, A., Perlmutter, H. and Davies, H. (1975) LKB Application Note 198
22 Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) Nature 195, 281—283
23 Swank, R.T. and Munkres, K.D. (1971) Anal. Biochem. 39, 462—477
24 Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 97, 79—87
25 De Belleroche, J.S. and Bradford, H.F. (1977) J. Neurochem. 29, 335—343
26 Wernicke, J.F., Oberjat, T. and Howard, B.D. (1974) J. Neurochem. 22, 781—788
27 Sen, I., Grantham, P.A. and Cooper, J.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2664—2668
28 Bergmeyer, H.U. (1965) Methods of Enzymatic Analysis, pp. 736, Academic Press, New York
29 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
30 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
31 MacDermot, J., Westgaard, R.H. and Thompson, E.J. (1978) Biochem. J. 175, 271—279
32 Kelly, R.B. and Brown, F.R. (1974) J. Neurobiol. 5, 135—150
33 Kondo, K., Narita, K. and Lee, C.Y. (1978) J. Biochem. 83, 91—99
34 Hanley, M.R., Eterović, V.A., Hawkes, S.P., Hebert, A.J. and Bennett, E.L. (1977) Biochemistry 16, 5840—5849
35 Chang, C.C., Chen, T.F. and Lee, C.Y. (1973) J. Pharmacol. Exp. Ther. 184, 339—345
36 Yamamura, H.I. and Snyder, S.H. (1972) Science 178, 626—628
37 Wernicke, J.F., Vanker, A.D. and Howard, B.D. (1975) J. Neurochem. 25, 483—496
38 Kondo, K., Narita, K. and Lee, C.Y. (1978) J. Biochem. 83, 101—115
39 Diniz, C.R. (1978) in Handbook of Experimental Pharmacology (Bettini, S., ed.), Vol. 48, pp. 379—394, Springer-Verlag, Berlin and Heidelberg
40 Barnard, E.A., Dolly, J.O., Lang, B., Lo, M. and Shorr, R.G. (1979) Advances in Cytopharmacology (Ceccarelli, B. and Clementi, F., eds.), Vol. 3, pp. 409—435, Raven Press, New York, NY
41 Langer, S.Z., Adler-Graschinsky, R., Almeida, A.P. and Diniz, C.R. (1975) Naunyn-Schmiedeberg's Arch. Pharmacol. 287, 243—259
42 Dowdall, M.J., Fohlman, J.P. and Eaker, D. (1977) Nature 269, 700—702
43 Martin, D.L. (1976) in GABA in Nervous System Function (Robert, E., Chase, T.N. and Tower, D.B., eds.), pp. 347—386, Raven Press, New York,
44 Catterall, W.A. (1977) J. Biol. Chem. 252, 8660—8668
45 Sen, I. and Cooper, J.R. (1978) J. Neurochem. 30, 1369—1375
46 Alderdice, M.T. and Volle, R.L. (1978) J. Pharmacol. Exp. Ther. 205, 58—68
47 Oberg, S.G. and Kelly, R.B. (1976) Biochim. Biophys. Acta 433, 662—673
48 Strong, P.N. and Kelly, R.B. (1977) Biochim. Biophys. Acta 469, 231—235
49 Ng, R.H. and Howard, B.D. (1978) Biochemistry 17, 4978—4986
50 Kelly, R.B., Oberg, S.G., Strong, P.N. and Wagner, G.M. (1975) Cold Spring Harbor Symp. Quant. Biol. 40, 117—125