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Beta-Bungarotoxin

SEPARATION OF TWO DISCRETE PROTEINS WITH DIFFERENT SYNAPTIC ACTIONS

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β-Bungarotoxin, a specific presynaptic blocking agent, was prepared in two stages from the crude venom of *Bungarus multicinctus* by ion-exchange chromatography on the weakly acidic ion exchanger, CM-Sephadex, and on the strongly acidic ion exchanger, sulphopropyl-Sephadex. By these procedures it was purified to a single protein, which was shown by reduction to contain two polypeptide chains with mol.wts. of less than 15000. During purification of β-bungarotoxin three other proteins were isolated. Two of these proteins have similar molecular weights, subunit structure and physiological properties to the major protein component. This latter is referred to as β-bungarotoxin, since it has the same physiological properties as those described for unpurified β-bungarotoxin by other workers. The first protein has very different physiological effects and biochemical properties from β-bungarotoxin. This protein has a single class of polypeptide chains with an apparent molecular weight that is lower than the main β-bungarotoxin protein, and appears to block synaptic transmission by a predominantly postsynaptic effect. It has been suggested [Oberg & Kelly (1976) *J. Neurobiol.* **7**, 129–141] that the action of β-bungarotoxin depends on its phospholipase A activity; however, in this preparation of the toxin less than 50 μunits of phospholipase A activity were detected (1 unit of activity is the amount of enzyme forming 1 μmol of L-α-phosphatidylcholine/min per mg of protein).

A constituent protein of the crude venom of *Bungarus multicinctus* named α-bungarotoxin has been shown (Chang & Lee, 1963) to produce a curare-like neurotoxic effect. Studies of ^{125}I-labelled α-bungarotoxin led to the isolation and characterization of the acetylcholine-receptor molecule (Changeux *et al.* 1970; Barnard *et al.*, 1971; Miledi & Potter, 1971; Schmidt & Raftery, 1973; Brockes & Hall, 1975). This work depended on the fact that α-bungarotoxin binds almost irreversibly to the acetylcholine receptor and, in addition, ^{125}I-labelled α-bungarotoxin retains its biological activity. Chang *et al.* (1973) suggested that studies of the presynaptic neurotoxin, β-bungarotoxin, might yield complementary results to those of α-bungarotoxin, if β-bungarotoxin were to bind to a specific macromolecule(s) involved in transmitter release by motor nerves. The possibility that the mechanism of acetylcholine release from nerve terminals might be further elucidated has stimulated the interest in β-bungarotoxin.

The original terminology of β-bungarotoxin and γ-bungarotoxin for the two presynaptic neurotoxins isolated by zone electrophoresis on starch (Chang & Lee, 1963) was abandoned when it was shown that the crude venom of *B. multicinctus* contained at least five presynaptic neurotoxins (Lee *et al.*, 1972). These fractions were separated by ion-exchange chromatography on CM-Sephadex C-50, the proteins were eluted with an ammonium acetate/acetic acid buffer of increasing ionic strength and pH from 50 mM, pH 5.0, to 1.0 M, pH 6.8. The term β-bungarotoxin was then confined to the most abundant of the presynaptic neurotoxins.

To date, other methods used in the preparation of β-bungarotoxin have all involved chromatography of the weakly acidic ion exchangers CM-Sephadex (Kelly & Brown, 1974; Dryden *et al.*, 1974) or CM-cellulose (Wernicke *et al.*, 1974). The protein fractions were eluted from the column in each case with a gradient of ammonium acetate/acetic acid buffer similar to, or identical with, that used by Lee *et al.* (1972). Both Lee *et al.* (1972) and Strong *et al.* (1976) have shown that β-bungarotoxin may be further purified by rechromatography on CM-cellulose.

It has been claimed that by these procedures a single protein is isolated in the preparation of β-bungarotoxin (Kelly & Brown, 1974). However, others have found that there are at least two constituent proteins in the final preparation of the toxin (Lee *et al.*, 1972; Wernicke *et al.*, 1974), as demonstrated by electrophoresis on cellulose acetate at pH 7.4.

The purpose of this investigation was to isolate pure β-bungarotoxin from the crude venom of *B. multicinctus* and characterize some of its physical,

Abbreviation used: SDS, sodium dodecyl sulphate.

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biochemical and physiological properties. Certain further information on methodology and results is given by MacDermot (1977).

Although full physiological details will be published elsewhere (R. H. Westgaard, D. N. Landon, J. MacDermot & E. J. Thompson, unpublished work) it is clear that work on β-bungarotoxin (fraction V of the crude venom) must be viewed with some caution, since others have detected at least two proteins and we have further separated four proteins from fraction V, two of which have quite different synaptic actions.

Experimental

Materials

Freeze-dried venom of *B. multicinctus*, phospholipase A from *Vipera russellii* and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (synthetic lecithin) were obtained from the Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. Sephadex was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide and Folin & Ciocalteu's phenol reagent were obtained from BDH Chemicals, Enfield, Middx., U.K. *NN'*-Methylenebisacrylamide was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. All chemicals used were of analytical grade.

Animals

White mice, N.I.H. strain DB5, were used.

Methods

Chromatography on CM-Sephadex C-25. A column (84cm × 1.5cm) of CM-Sephadex C-25 was equilibrated with 50mM-ammonium acetate/acetic acid buffer, pH5.8, at room temperature (20°C). Thereafter 2ml of the same buffer solution containing 50mg of whole venom of *B. multicinctus* was applied to the top of the column and the proteins were eluted with an ammonium acetate/acetic acid buffer gradient of increasing ionic strength and pH. The initial gradient was prepared with 250ml of 50mM-ammonium acetate/acetic acid buffer, pH5.8, and 250ml of 0.5M-ammonium acetate/acetic acid buffer, pH7.0. After the emergence of peak 2 (Fig. 1), the gradient was changed and was thereafter prepared with 250ml of 0.5M- and 250ml of 1.0M-ammonium acetate/acetic acid buffer, pH7.0.

Fractions (5ml) were collected at a flow rate of 0.4–0.6ml/min. The proteins eluted from the column were identified in the individual fractions by measurement at A_{280} in a 1cm-path-length cell in a spectrophotometer (Unicam SP.500; Pye–Unicam, Cambridge, U.K.). The osmolarity of selected fractions was also recorded in an osmometer (model 3W, Advanced Instruments, Newton Highlands, MA, U.S.A.).

Chromatography on SP (sulphopropyl)-Sephadex. A column (15cm × 1.5cm) was prepared of SP-Sephadex C-25 equilibrated with 50mM-ammonium acetate/acetic acid buffer, pH5.0 at room temperature. The crude β-bungarotoxin (peak 5 in Fig. 1) was diluted with water until the osmolarity was 200mosM. The solution was then acidified with acetic acid to pH5.0 and applied to the top of the column at a flow rate of 0.4–0.6ml/min. The proteins were eluted from the column in 50mM-ammonium acetate/acetic acid buffer, pH5.0, containing a NaCl gradient of increasing ionic strength from 0.21M to 0.5M. The gradient was prepared with 250ml of each solution. Fractions (5ml) were collected at a flow rate of 0.4–0.6ml/min and the proteins were identified in the individual fractions as before. The osmolarity of selected fractions was again recorded.

Electrophoresis on cellulose acetate at pH7.4. Cellulose acetate strips (Shandon Celogram SAE-226, Shandon, Camberley, Surrey, U.K.) (15cm × 7.8cm) were prepared by soaking them in 0.1M-sodium phosphate buffer, pH7.4, for 5 min. Samples were applied to the surface of the strip in a line about 0.5cm long, at right angles to the direction of the current, midway between the anode and cathode. The electrode solution at either end of the strip was 0.1M-sodium phosphate buffer, pH7.4, and a current of 6mA was passed for a total of 2h at room temperature. The cellulose acetate strip was then stained for 30min in 0.25% (w/v) Coomassie G-250 and destained in 7% (v/v) acetic acid, containing 10% (v/v) methanol.

Electrophoresis on agarose at pH7.4. An agarose plate (ACL Universal, Corning–Eel, Halstead, Essex, U.K.) (width 12.5cm × length 11.5cm × depth 0.6mm) was equilibrated with 0.1M-sodium phosphate buffer, pH7.4, by prerunning the plate for 1h at a current of 10mA. The electrode solution at either end of the plate was 0.1M-sodium phosphate buffer, pH7.4. Samples were then loaded into wells (6mm × 1mm) cut in the agarose, situated two-thirds of the way down the plate from the anode. The samples were run in renewed electrode solutions at room temperature for 2h at 10mA. The agarose plates were stained by the procedure used for the cellulose acetate strips.

Polyacrylamide-gel electrophoresis. Polyacrylamide gels were prepared (8%, w/v, with 1:30, w/v, *NN'*-methylenebisacrylamide) in glass columns (9cm × internal diam. 0.5cm.) (Davis, 1964). Protein samples were applied in 25μl to the upper surface of the gel and the electrophoresis was performed at 6mA/tube for 2h at room temperature. The electrode solution at both ends of the gels was 0.1M-sodium phosphate buffer, pH7.0. The cathode was located at the lower end of the gels. The gels were removed from the glass tubes and stained for 2h in 0.25% (w/v) Coomassie G-250 and then destained in 7% (v/v) acetic acid, containing 10% (v/v) methanol.

SDS/polyacrylamide-gel electrophoresis. This

electrophoresis was performed by the method of Weber & Osborn (1969). Polyacrylamide gels (8%, w/v, with 1:30, w/v, *NN'*-methylenebisacrylamide) were prepared in glass columns (9cm × internal diam. 0.5cm). Protein samples were incubated in 25μl volumes with SDS and 2-mercaptoethanol by the method of Weber & Osborn (1969), or alternatively in similar incubations without the 2-mercaptoethanol. The electrode solution at either end of the gels was 50mM-sodium phosphate buffer, pH7.2, containing 0.1% (w/v) SDS and electrophoresis was performed at 6mA/tube for 2.75h at room temperature. The gels were stained and destained as described above.

Gel scanning. The gels were scanned on a chromoscanning densitometer (Roboscan, Joyce–Loebl, Gateshead, Tyneside, U.K.).

Protein concentration. Protein concentration was determined by the method of Lowry *et al.* (1951), with dilutions of bovine serum albumin as the standard solutions.

Phospholipase A enzymic activity. The method used depended on the ability of the enzymic reaction product of phospholipase A, lysophosphatidylcholine to disrupt erythrocytes, as described by Wernicke *et al.* (1975). The standard curve was obtained with phospholipase A from *V. russellii* with a specific activity of 11.0 units of enzymic activity (1 unit of activity forms 1μmol of L-α-phosphatidylcholine/min per mg of protein) at concentrations of 0.31–5.0μg ml. Crude β-bungarotoxin and each of its four constituent fractions were also tested for phospholipase A activity at various concentrations up to 100μg/ml. A control buffer blank was also included and all samples were assayed in duplicate.

Physiological methods

The soleus muscle and its nerve supply were removed from both legs of rats (weight 150–250g) under Nembutal (May and Baker, Dagenham, Essex, U.K.) anaesthesia. The muscles were placed in a Perspex bath (volume 15ml) filled with mammalian Ringer's solution at room temperature. The Ringer's solution contained in most experiments 137mM-NaCl, 4mM-KCl, 5mM-$CaCl_2$, 1mM-$MgCl_2$, 18mM-$NaHCO_3$, 1mM-KH_2PO_4, 10mM-D-glucose. It was continually oxygenated with O_2/CO_2 (19:1). The pH was 7.15–7.25 throughout the experiment. O_2 partial pressure in the solution was more than 53kPa. In the experiments with increased [Ca^{2+}] (5mM), albumin (15mg/ml) was added to the bath to avoid non-specific binding of the protein fractions. The concentration of Ca^{2+} not bound to proteins was 3mM in these experiments. In some experiments albumin was not added to the bath and a decreased concentration of Ca^{2+} (2mM) was then used. There was no apparent change in the physiological effects of the protein fractions with this modification of the bathing solution.

In a standard experiment one muscle was attached to a force transducer and the isometric twitch tension to nerve stimulation recorded. Miniature end-plate potentials were recorded intracellularly in surface fibres of the other, unstimulated muscle, with conventional micropipettes filled with potassium citrate (3M) and having resistances of 5–10MΩ. The isometric twitch tension was considered a valid measure of the efficiency of neuromuscular transmission, since it was established that direct stimulation of the muscle after exposure to either of the protein fractions resulted in a twitch tension that was similar to the initial tension; nor was there any decrease in the amplitude of the nerve action potential during the course of each experiment. In these experiments the rate of stimulation was 0.5Hz. At this frequency there was an initial fall in twitch tension for 30–60min. The twitch tension then remained stable at 50–70% of the original tension for the next 150–240min (three experiments). The initial decrease in tension may be due to lack of O_2 in the deep part of the muscle, and it was reversible in that the twitch tension recovered to its original value after a 1–2min pause in nerve stimulation.

The protein fractions were applied to the nerve–muscle preparation by diluting the appropriate amount of concentrated toxin (250–1000μg/ml) in 3–5ml of Ringer's solution, which was removed from the bath. The diluted toxin was then distributed evenly in the bath. The toxin remained in the bath for the duration of the experiment. The effect of the protein fractions 1 and 3 on the acetylcholine receptor was tested by measuring the influence of these protein fractions on the extrajunctional acetylcholine sensitivity of chronically denervated rat soleus muscle fibres with fully developed extrajunctional acetylcholine sensitivity (6–8 days denervated). The acetylcholine sensitivity was measured by iontophoretic application of acetylcholine chloride, the technique being that described by Lømo & Westgaard (1975). Sensitivity was expressed as millivolt of depolarization per nanocoulomb change passed through the acetylcholine pipette (Miledi, 1960). A denervated soleus muscle with fully developed extrajunctional acetylcholine sensitivity was maintained in a separate compartment to the muscle exposed to either of the toxins and was used to check the state of the acetylcholine pipette in the course of an experiment. If extrajunctional acetylcholine sensitivities of less than 100mV/nC were found in the denervated control muscle, the pipette was rejected and the measurements were repeated with a new pipette (Lømo & Westgaard, 1975).

Results

The isolation of crude β-bungarotoxin from the whole venom of *B. multicinctus* on CM-Sephadex

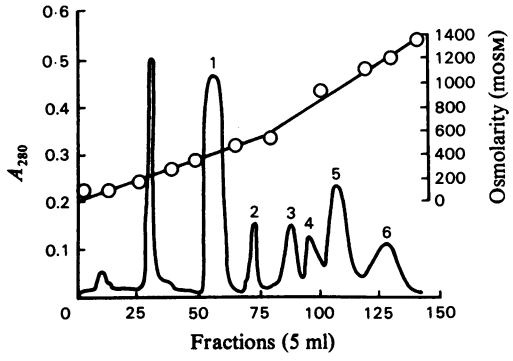

Fig. 1. *Chromatography of the venom of B. multicinctus* Ion-exchange chromatography of the venom of *B. multicinctus* on CM-Sephadex C-25. The proteins were eluted with an ammonium acetate/acetic acid buffer gradient (○) of increasing ionic concentration and pH from 50mM, pH5.8, to 1.0M, pH7.0. For details of peaks 1–6, see the text.

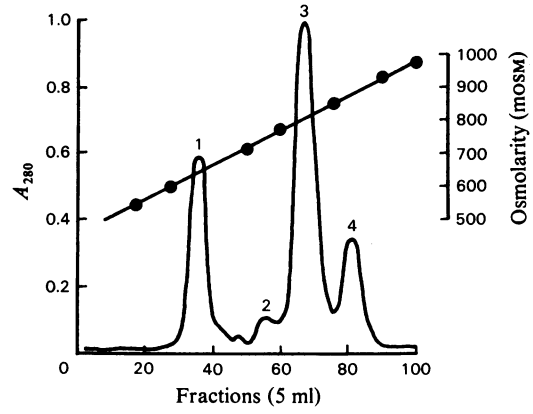

Fig. 2. *Chromatography of crude β-bungarotoxin* Ion-exchange chromatography of crude β-bungarotoxin on SP-Sephadex C-25. The proteins were eluted with a NaCl gradient (●; 0.21–0.5M) in 50mM-ammonium acetate/acetic acid buffer, pH5.0.

C-25 is shown in Fig. 1. The peaks of protein were labelled from 1 to 6, according to the numbering scheme of Kelly & Brown (1974). Peak 1 is known to be α-bungarotoxin (Lee *et al.*, 1972) and peak 5 has been identified as β-bungarotoxin (Kelly & Brown, 1974). A large peak was eluted from the column before the α-bungarotoxin, which has been shown to be guanosine (Lee *et al.*, 1972).

The β-bungarotoxin prepared in this way was further fractionated into four constituent proteins by ion-exchange chromatography on SP-Sephadex C-25 (Fig. 2).

Biochemical properties of protein fractions.

Electrophoretic examination of the toxins was performed on three different support media (Table 1). The β-bungarotoxin migrated as a single charged species with a greater net basic charge than α-bungarotoxin. The whole venom could be resolved into only four diffuse bands by electrophoresis on polyacrylamide gel and the middle two bands represented about 80% of the total protein applied. The two major bands were approximately equal in size after Coomassie Blue staining and scanning of the gel.

Examination of the four constituent proteins of crude β-bungarotoxin was performed by polyacrylamide-gel electrophoresis in the presence of SDS. Under non-reducing conditions, all four proteins migrated as single bands (Fig. 3), but after the protein samples had been reduced with 2-mercaptoethanol, three of the four samples were shown to be made up of two polypeptide subunits. Comparison of the migration distances of these subunits with known molecular-weight standards showed that peak 1 and the subunits of peaks 2, 3 and 4 all have molecular weights apparently below 15000.

The crude β-bungarotoxin and its four constituent proteins were examined for phospholipase A activity. The standard curve of this assay is presented in Fig. 4. At protein concentrations of up to 100μg/ml neither the crude β-bungarotoxin nor any of its four constituents had more than twice the enzymic activity of the buffer blank. It thus follows that β-bungarotoxin contains less than 50μunits of enzymic activity (1 unit = 1μmol of phosphatidylcholine formed/min per mg of protein). The toxicity of the β-bungarotoxin was tested by intravenous injection into the tail vein of a mouse and was in the same range as that described by Lee *et al.* (1972) and Strong *et al.* (1976). The LD_{50} was 0.01–0.03μg/g body wt. The characteristic muscular paralysis that follows an initial stage of hyperexcitability (Chang & Lee, 1963) was observed. For comparison, the toxicity of phospholipase A from *V. russellii* was tested by intravenous injection. The neuromuscular paralysis characteristic of β-bungarotoxin was not observed and a dose of 1.5μg/g body wt. was not lethal.

Physiological effects of protein fractions

The effect of protein fraction 3 on synaptic transmission was measured indirectly by the contractile force of isometric twitch responses to single nerve stimuli (see under 'Methods'). A typical result from such an experiment is shown in Fig. 5. The muscle twitch response in Fig. 5 showed a fall during the first 20min after application of the protein, followed by a relative facilitation and thereafter a slow decline in tension over the next 4h. The size of the initial fall and the subsequent facilitation varied from experiment to

Table 1. *Electrophoretic examination of the whole venom of B. multicinctus, α-bungarotoxin and β-bungarotoxin on cellulose acetate, agarose and polyacrylamide gel*

Electrophoresis on cellulose acetate and agarose was performed for 2h at room temperature with a current of 6mA and 10mA respectively. The electrode solution was 0.1M-sodium phosphate buffer, pH7.4, in each case. Electrophoresis on polyacrylamide-gel columns was performed at room temperature for 2h at 6mA/tube. The electrode solution was 0.1M-sodium phosphate buffer, pH7.0.

Support medium	Material	Amount applied (μg)	pH	Distance migrated towards the cathode (cm)
Cellulose acetate	α-Bungarotoxin	6	7.4	0.7
	β-Bungarotoxin	2	7.4	0.8
Agarose	α-Bungarotoxin	6	7.4	0.6
	β-Bungarotoxin	2	7.4	0.7
	α-Bungarotoxin + β-bungarotoxin	6 + 2	7.4	0.6, 0.7
Polyacrylamide gel	Whole venom	25	7.0	2.3, 1.8, 1.3, 0.3
	α-Bungarotoxin	16	7.0	1.3
	β-Bungarotoxin	5	7.0	1.8
	α-Bungarotoxin + β-bungarotoxin	6 + 3	7.0	1.3, 1.8

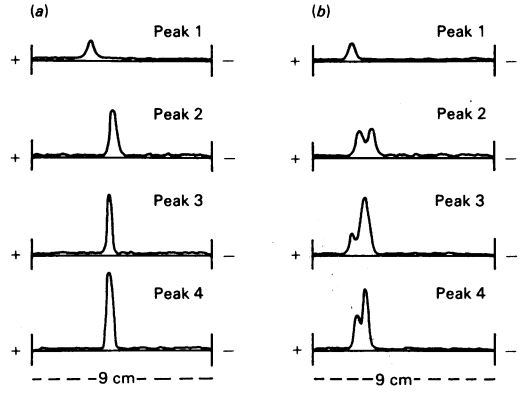

Fig. 3. *Electrophoresis of the four constituent proteins of crude β-bungarotoxin on polyacrylamide gels under reducing and non-reducing conditions*

Densitometer scans of polyacrylamide gels are shown. Electrophoresis was carried out at pH7.2 in the presence of SDS under non-reducing conditions (*a*) and under reducing conditions with 2-mercaptoethanol (*b*).

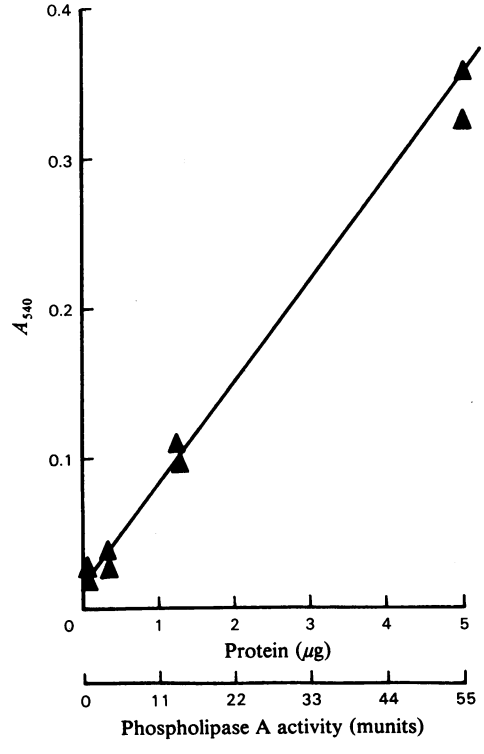

Fig. 4. *Assay of phospholipase A*

The A_{540} in a 1cm-path-length cell was measured after lysis of erythrocytes by lysophosphatidylcholine produced by the action of various concentrations of phospholipase A on phosphatidylcholine. All measurements were made with water in the reference cell. Additional experimental details are provided in the text.

experiment, but the time to complete a block of twitch tension was at least 2½h in eight experiments with concentrations of fraction 3 of between 3 and 10μg/ml, and the time was even longer with lower concentrations of the toxin.

Protein fraction 3 also has a marked effect on the spontaneous release of synaptic transmitter, as indicated by the generation of miniature end-plate potentials in the muscle fibre. Fig. 6 shows the rate of generation of these potentials in a muscle fibre of an unstimulated muscle exposed to protein fraction 3

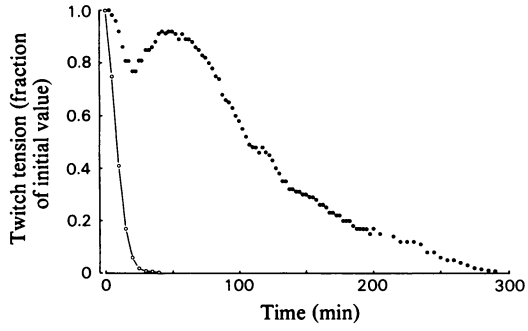

Fig. 5. *Effect of β-bungarotoxin fraction* 1 *and fraction* 3 *on isometric twitch response*
Isometric twitch responses to nerve stimulation of rat soleus muscles, exposed to protein fraction 1 (10 μg/ml, ○) or to fraction 3 (3 μg/ml, ●). The twitch responses of the two muscles have been normalized and are plotted as a function of time (min) after application of the protein to the bath. The Ringer solution contained albumin (15mg/ml) and an increased concentration of Ca^{2+} (5mM, see under 'Methods'). Twitch tension is expressed in arbitrary units (ordinate) as a fraction of the initial tension developed when toxin was added to the bath (1.0).

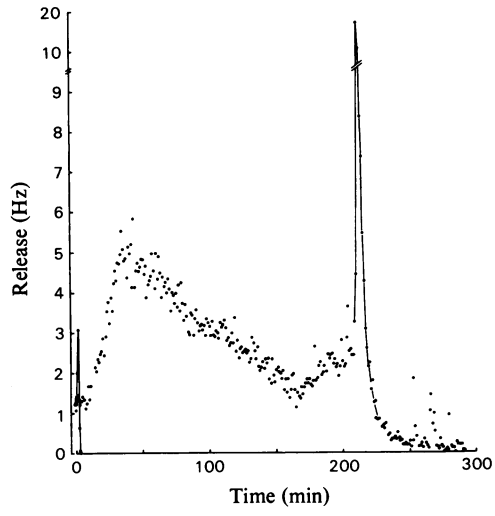

Fig. 6. *Effect of β-bungarotoxin fraction* 1 *and fraction* 3 *on spontaneous release of miniature end-plate potentials*
Spontaneous release of miniature end-plate potentials from single end plates of unstimulated soleus muscles maintained in the Perspex bath and exposed to protein fraction 1 (10 μg/ml, ○) and fraction 3 (3 μg/ml, ●). These muscles were maintained and studied simultaneously with the muscles used to produce the data in Fig. 5. The rate of release (Hz) is plotted as a function of time (min) after toxin application. The points on the graph represent average rate of release over 1 min periods (●, fraction 3) or 30s periods (○, fraction 1). Individual points are joined by lines during the late burst (at 220min) with rapid release of miniature end-plate potentials after exposure to protein fraction 3.

simultaneously with the stimulated muscle illustrated in Fig. 5. The rate of generation of these end-plate potentials in this fibre increased 4-fold over the first 40min, followed by a slow decline that was again reversed after 165min, when there was a gradual increase culminating in a short period of very rapid generation of the potentials. Thereafter the rate of generation fell rapidly to a very low value. The early increase in the rate of generation of miniature end-plate potentials (peak frequency 2–10 times the initial frequency) was observed in more than 20 experiments with the concentration of protein fraction 3 ranging from 0.3 to 10 μg/ml. The late burst of these potentials is a feature in a majority of the experiments where the full time course of their generation was followed in a single muscle fibre.

Prolonged exposure of the nerve–muscle preparation to protein fraction 3 results in the generation of these potentials with decreased amplitude. This is illustrated in Figs. 7(*a*), 7(*b*) and 7(*c*), which show the combined amplitude histogram of miniature end-plate potentials from six muscle fibres before application of protein fraction 3 (Fig. 7*a*) and from two groups of six different fibres in the same muscle after exposure to the toxin for 65–85min (Fig. 7*b*) and 130–170min (Fig. 7*c*). Although potentials of normal size were present in all muscle fibres throughout the experiment, it is clear that these small-amplitude potentials are much more frequent in the later stage of this experiment.

This phenomenon is unlikely to be of postsynaptic origin, since incubation with 10 μg of protein fraction 3/ml for 2h results in very little or no decrease in the sensitivity of chronically denervated muscle fibres to iontophoretically applied acetylcholine chloride (Fig. 8*b*). These results and the fact that miniature end-plate potentials with normal amplitude remain after prolonged exposure to protein fraction 3 make it likely that the physiological effects of this toxin are predominantly of presynaptic origin, despite the appearance of some miniature end-plate potentials with decreased amplitudes.

Exposure of the nerve–muscle preparation to protein fraction 1 results in a rapid fall of twitch tension, the time course being dependent on the concentration used. At a concentration of 10 μg/ml, the twitch tension was abolished 40–60min after toxin application (three experiments) (Fig. 5). The effect of the toxin on end-plate potentials in surface fibres is even more rapid, with the end-plate potentials being

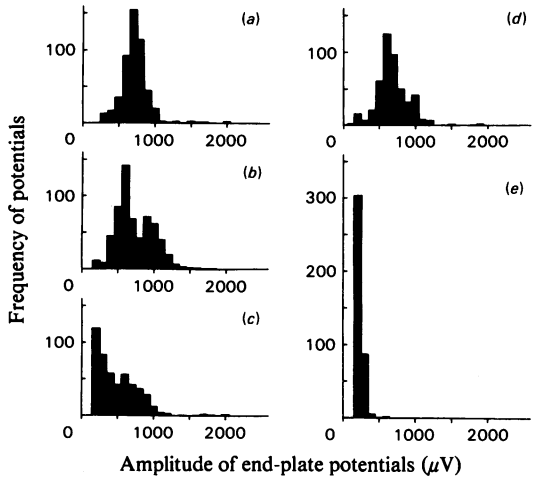

Fig. 7. *Effect of protein fraction* 3 (*a, b, and c*) *and fraction* 1 (*d and e*) *on the amplitude of miniature end-plate potentials*
The Figure shows an amplitude histogram of miniature end-plate potentials from six muscle fibres before application of neurotoxin (*a*), from six (different) fibres in the same muscle 65–85 min after application of protein fraction 3 (3 μg/ml; *b*) and from another six fibres in the same muscle 130–170 min after toxin application (*c*). Mean amplitude of the end-plate potentials from the individual fibres was in the range 634–872 μV (*a*); 524–1023 μV (*b*); and 412–592 μV. (*c*) The lowest amplitude that could be separated from noise was 100 μV in this experiment. In a separate experiment the effect of protein fraction 1 on these potential amplitudes was tested. The Figure shows the amplitude histogram from seven muscle fibres before (*d*), and from six (different) muscle fibres 80–120 min after, (*e*) application of protein fraction 1 (3 μg/ml). Mean amplitude of these potentials from the individual fibres was in the range 550–900 μV before application of fraction 1 (*d*) and 120–150 μV afterwards (*e*). The lowest amplitude detected in this experiment was 50 μV. No albumin was added to the Ringer's solution in these experiments and the Ca^{2+} concentration was 2 mM. Mean rise time of these end-plate potentials (i.e. from 25% of maximal amplitude to maximal amplitude) was less than 1.5 ms for all fibres.

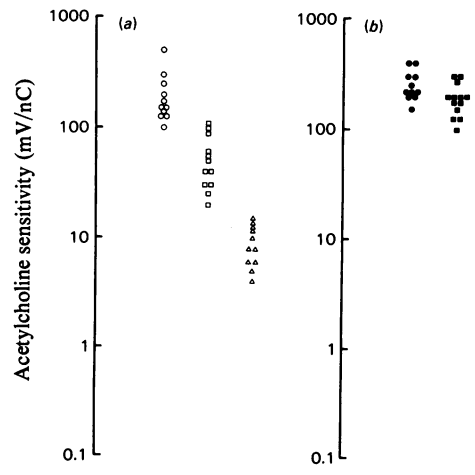

Fig. 8. *Acetylcholine sensitivities of individual muscle fibres to iontophoretically applied acetylcholine chloride in two rat soleus muscles, both denervated for* 7 *days*
The acetylcholine chloride was applied 3–5 mm away from the position of the denervated end plate. (*a*) ○, Untreated denervated muscle fibres; □, fibres from same muscle 1 h after application of protein fraction 1 (3 μg/ml); △, fibres from same muscle 1 h after the concentration of protein fraction 1 was increased to 10 μg/ml. (*b*) ●, Untreated denervated muscle fibres from another muscle; ■, muscle fibres from the second denervated muscle 1.2 h after application of protein fraction 3 (10 μg/ml). No albumin was added to the Ringer solution; Ca^{2+} concentration was 2 mM.

substantially decreased or completely abolished within 5–10 min of toxin application (R. H. Westgaard, D. N. Landon, J. MacDermot & E. J. Thompson, unpublished work).

The effect of protein fraction 1 on the miniature end-plate potentials is equally rapid, with the potentials disappearing within 5 min after application of 10 μg of the toxin/ml (Fig. 6). In some fibres a decrease in the size of these potentials was observed as these potentials disappeared, and when lower concentrations of protein fraction 1 were used, clear end-plate potentials with very decreased amplitudes were observed throughout the experiment. Figs. 7(*d*) and 7(*e*) show the amplitude histogram of these potentials from six muscle fibres before application of 3 μg of protein fraction 1/ml (Fig. 7*d*) and six different fibres from the same muscle 80–120 min after toxin application (Fig. 7*e*). It is clear that the size of all miniature end-plate potentials is decreased relative to that in normal muscle fibres after exposure to this protein fraction. When protein fraction 1 is applied to chronically denervated muscle, there is a very clear dose-dependent decrease in the sensitivity of the muscle membrane to iontophoretically applied acetylcholine (Fig. 8). From these results it appears that protein fraction 1 has a predominantly postsynaptic action. Protein fractions 2 and 4 have physiological effects that are very similar to those of protein fraction 3. However, one should bear in mind that these fractions are likely to be slightly contaminated by fraction 3.

Discussion

Lee *et al.* (1972) have shown that crude β-bungarotoxin may be purified on CM-cellulose. By this procedure, two minor peaks of materials, followed by

an irregularly shaped major peak of protein, were eluted from the column. These fractions might well correspond to the four peaks identified in Fig. 2, there being little separation of peaks 3 and 4 on CM-cellulose. The same group of workers found, in addition, that their preparation of β-bungarotoxin contained two proteins that could be identified by electrophoresis on cellulose acetate at pH7.4. The alternative procedure is to perform ion-exchange chromatography on the strongly cationic exchanger SP-Sephadex, which would then separate β-bungarotoxin from its impurities more effectively. The β-bungarotoxin prepared by this procedure migrated as a single band on cellulose acetate and agarose at pH7.4. Similar electrophoresis on polyacrylamide at pH7.0 also identified only one charged species. Electrophoresis of the whole venom on polyacrylamide demonstrated only four rather diffuse bands, which are probably the four fractions originally identified by Chang & Lee (1963) by zone electrophoresis on starch.

Our fractions 1, 2, 3 and 4 are not to be confused with the β_1, β_2, β_3 and β_4 fractions of Abe *et al.* (1977). The latter investigators have confirmed and extended the findings of Lee *et al.* (1972), namely, that the proteins were presynaptic in their mode of action. The appropriate numbers from Lee *et al.* (1972) were IV_1, IV_2, V and VII respectively (see their Table III). We have found four proteins from the fraction V of Lee *et al.* (1972) [or from β_3 of Abe *et al.* (1977)], one of which has a postsynaptic action. The observations of the subunit structure of β-bungarotoxin confirm the findings of Kelly & Brown (1974). The molecular weights of the two subunits of β-bungarotoxin are, however, only presented as being less than 15000 in each case as this represents the lower limit of reliability for this method of molecular-weight determination (Fish *et al.*, 1970).

It has been suggested that the neurotoxic effects of β-bungarotoxin depend on its phospholipase A activity (Wernicke *et al.*, 1975; Howard, 1975; Kelly *et al.*, 1975; Abe *et al.*, 1976), and Strong *et al.* (1976) have shown that the putative phospholipase A activity is dependent on the presence of the detergent sodium deoxycholate. The preparation of β-bungarotoxin described here retained the characteristic neurotoxic properties ascribed to it, but had no detectable phospholipase A activity in the absence of detergent. This confirms the work of Lee *et al.* (1972), who could not demonstrate phospholipase A activity in their preparation of β-bungarotoxin.

Injection of phospholipase A from *V. russellii* did not produce the muscular paralysis characteristic of β-bungarotoxin. This would seem to support the finding of Strong *et al.* (1976), who showed that phospholipase A from *V. russellii* does not produce the increase in frequency of the spontaneous release of acetylcholine, which is a feature of β-bungarotoxin. The question of the association of the neurotoxic properties of β-bungarotoxin with phospholipase A activity has been further studied by Sen *et al.* (1976), who showed that neither the release of transmitter nor the inhibition of choline uptake produced by the toxin was affected by the presence of an inhibitor of phospholipase A activity. When phospholipase activity was assayed with a bacterial membrane preparation the standard enzyme was, if anything, stimulated by the same inhibitor (Ng & Howard, 1977).

The neurotoxic effects of protein fraction 1 are very different from β-bungarotoxin. Protein fraction 3 has been shown to have similar physiological properties to those described for the unfractionated β-bungarotoxin described by other workers (Lee & Chang, 1966; Chang *et al.*, 1973; Kelly & Brown, 1794; Abe *et al.*, 1976), and it therefore appears reasonable to refer to this fraction as β-bungarotoxin. Earlier work and the results of the present paper indicate that β-bungarotoxin has a predominantly presynaptic effect. The appearance of miniature end-plate potentials with decreased amplitude after prolonged exposure to β-bungarotoxin is therefore puzzling, but may be understood in view of a report indicating that the uptake of choline in peripheral cholinergic endings is decreased after exposure to β-bungarotoxin (Dowdall *et al.*, 1977). It may therefore represent the effect of release of acetylcholine from partially filled synaptic vesicles.

The neurotoxic effects of protein fraction 1 are very different from β-bungarotoxin. The results in the present paper indicate that this toxin blocks neuromuscular transmission by a predominantly postsynaptic action.

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