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Beta-Bungarotoxin

THE BINDING OF [^{3}H]PYRIDOXYLATED BETA-BUNGAROTOXIN TO A HIGH-MOLECULAR-WEIGHT PROTEIN RECEPTOR

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β-Bungarotoxin was labelled with pyridoxal 5′-phosphate (incorporating ^{3}H). The kinetics of β-bungarotoxin binding to several tissue subfragments of nervous tissue was studied. The dissociation constant of ^{3}H-pyridoxylated β-bungarotoxin in this reaction was 0.21–0.37 μM and that of unlabelled β-bungarotoxin was 25 nM. Hill [(1910) *J. Physiol. (London)* **40**, iv–vii] and Scatchard [(1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672] analyses demonstrated no co-operativity of binding and only a single class of receptor sites, consistent with a bimolecular association of β-bungarotoxin and its receptor. The iodinated toxin was physiologically inactive. Toxin was bound in non-specific unsaturable fashion by glass and/or plastic. This low-affinity binding was corrected by addition of bovine serum albumin to a final concentration of 30 mg/ml. A soluble protein receptor of β-bungarotoxin was isolated and the mol.wt. is approx. 200000.

β-Bungarotoxin has been labelled with ^{125}I (Oberg & Kelly, 1976), and saturable binding to cell membranes from brain, liver and erythrocytes was demonstrated. Little or no difference was found in the dissociation constant (K_d) for the binding of ^{125}I-labelled β-bungarotoxin to these fragments (0.7–2.2 nM), although the density of receptor was greatest in the brain membrane fragments. Further analysis of the binding to brain membranes showed that the receptor density was higher in the 'low-density fragments' (crude myelin preparation) than in either the synaptosomal or the mitochondrial fractions. Oberg & Kelly (1976) were unable to isolate a receptor from the membrane preparations and were thus unable to resolve whether or not the saturable binding was specific to a protein receptor. It has been suggested that the neurotoxic properties of β-bungarotoxin depend on its phospholipase A activity (Wernicke *et al.*, 1975; Howard, 1975; Kelly *et al.*, 1975; Strong *et al.*, 1976). This would presuppose that the membrane receptor is a lipid. Since it would be exceptional for a lipase (or any other enzyme) to bind to its substrate with a K_d of 0.7–2 nM, it was suggested by Oberg & Kelly (1976) that β-bungarotoxin has two binding sites, one a phospholipase A site and the other a high-affinity site for an appropriate membrane receptor.

The purpose of these experiments was to extend the studies of the binding kinetics of radioactively labelled β-bungarotoxin to its receptor and, if possible, isolate and characterize the receptor itself.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Materials

Myosin (from striated muscle) was a gift from the Department of Biophysics, King's College, Drury Lane, London, U.K. The NaB^3H_4 and a [^{3}H]toluene standard were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Acrylamide and Folin–Coicalteu's phenol reagent were obtained from BDH Chemicals, Enfield, Middx., U.K. The *NN′*-methylenebisacrylamide was obtained from Eastman Kodak Co., Rochester, NY, U.S.A. The *NN′*-diallyltartardiamide was obtained from Serva, Heidelberg, Germany. The 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were supplied by Nuclear Enterprises, Edinburgh, U.K. Triton X-100 was supplied by Packard Instrument Co., Downers Grove, IL, U.S.A. Pyridoxal 5′-phosphate was obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Animals

White Wistar rats and white mice, N.I.H. strain DB5, were used.

Methods

Preparation of scintillant. Scintillant (100 ml) was prepared by the addition of 25 ml of Triton X-100 to 75 ml of toluene containing 0.4 g of PPO/100 ml and 0.01 g of POPOP/100 ml. The scintillant was mixed well and stored in the dark.

Pyridoxal 5'-phosphate labelling of β-bungarotoxin. A column (12cm×0.9cm) of Sephadex C-25 was prepared at room temperature by using Sephadex equilibrated with 5mM-sodium phosphate buffer, pH7.0. β-Bungarotoxin was prepared from the venom of *Bungarus multicinctus* by the method of MacDermot *et al.* (1978). The labelling procedure was performed in a hood with adequate air extraction, by a modification of the method described by Churchich (1965). A 1.6ml incubation mixture, in a 2ml plastic tube, contained 31.2μM-β-bungarotoxin, 0.51mM-pyridoxal 5'-phosphate, 1.0M-NaCl and 30mM-Na_2HPO_4, pH8.5. This was incubated at 37°C for 90min and then cooled to 4°C in an ice bath. A sample (3μl) of NaB^3H_4 (sp. radioactivity 6Ci/mmol), freshly prepared at 4°C, was added so that the final concentration of $NaBH_4$ in the incubation was 1.65mM. The incubation was left for a further 30min at 4°C and was then acidified with one drop of acetic acid. The incubation tube was shaken vigorously and gaseous ^3H was evolved. The pH was checked and was 5.0. The incubation was then diluted to a final volume of 50ml (to decrease the salt concentration) and this was loaded on the column at room temperature at a flow rate of 0.4–0.6ml/min. A total of 150ml of 5mM-sodium phosphate buffer, pH7.0, was then passed through the column to remove the excess pyridoxal 5'-phosphate and NaB^3H_4. A NaCl gradient of increasing ionic strength was then started to elute the protein from the column. The gradient was prepared from 2×200ml volumes of 5mM-sodium phosphate buffer, pH7.0, containing NaCl at concentrations of 50mM and 1.0M. The flow rate was maintained at 0.4–0.6ml/min and 2ml fractions were collected. The A_{280} of each fraction was measured in a 1cm-path-length cell in a spectrophotometer (Unicam SP.500, Pye–Unicam, Cambridge, U.K.) and the osmolarity of selected fractions was measured in an osmometer (model 3W, Advanced Instruments Inc., MA, U.S.A.). From every fifth fraction, a sample (50μl) was pipetted into 10ml of scintillant and these were counted for a time sufficient to give at least 10^4 counts in a scintillation counter (Packard Tri-Carb liquid-scintillation spectrometer, Packard Instrument Co.). Counting efficiencies were determined with a [^3H]toluene standard. Counting efficiencies (quenched) varied between 28 and 35%. A sample of β-bungarotoxin was also labelled with pyridoxal 5'-phosphate by an identical procedure, except that the reduction of the Schiff base was effected with non-radioactive $NaBH_4$. The preparation will be referred to as 'non-radioactive pyridoxal 5'-phosphate-labelled β-bungarotoxin'.

Preparation of synaptosomes and their subfragments. Synaptosomes were prepared from whole rat brain by the method of Gray & Whittaker (1962), modified by Whittaker *et al.* (1964). Synaptic vesicles were prepared by the method of Whittaker *et al.* (1964). Before their use for kinetic studies, these preparations were suspended in 5mM-sodium phosphate buffer, pH7.0, containing 150mM-NaCl and 30mg of bovine serum albumin/ml. This was carried out by washing 2ml of each suspension with 4×1ml of this solution (at 4°C) followed by centrifugation at 8000*g* ($r_{av.}$ 17cm) for 5min. Experiments carried out in the absence of bovine serum albumin showed that all of the synaptosomal protein was pelleted by this centrifugation. This fraction was used to derive the kinetic data.

Incubation for kinetic studies. Suspensions of the tissue fragments were incubated at room temperature in volumes of 200μl containing 5mM-sodium phosphate buffer, pH7.0, 150mM-NaCl, 30mg of bovine serum albumin/ml and ^3H-pyridoxylated β-bungarotoxin at the appropriate concentration. On completion of the incubation time, the reaction was terminated by centrifugation at 8000*g* ($r_{av.}$ 17cm) for 5min. The supernatant was discarded and the pellet washed with 6×1ml of 5mM-sodium phosphate buffer, pH7.0 (at 4°C), containing 150mM-NaCl and 30mg of bovine serum albumin/ml, followed by recentrifugation at 8000*g* ($r_{av.}$ 17cm) for 5min. Finally, 0.5ml of 10% (w/v) SDS was added to each tube and this was heated at 100°C for 3min in a water bath. Each solution was then added to 10ml of scintillant and the individual samples were counted in a scintillation counter for a time sufficient to give at least 10^4 counts.

Protein concentration. Protein concentration was determined by the method of Lowry *et al.* (1951), with dilutions of bovine serum albumin as standard. Protein estimations were performed on tissue-fragment samples that had been solubilized in 10% (w/v) SDS by heating at 100°C for 3min. For these determinations, the standard bovine serum albumin solutions contained the same concentration of SDS. There was, however, little difference in the standard curve as a result of the addition of SDS.

Polyacrylamide-gel electrophoresis. Rod gels (9cm ×internal diam. 0.5cm) were prepared by the method of Weber & Osborn (1969) with 8% (w/v) polyacrylamide containing 1:30 (w/w) *NN'*-methylenebisacrylamide. The gels were pre-run for 1h in 50mM-Tris/HCl buffer, pH8.3, containing 0.1% (w/v) SDS, at 6mA/tube at room temperature (20°C). Gels of the same size were also prepared with 8% (w/v) polyacrylamide, but with *NN'*-diallyltartardiamide substituted mol for mol in the place of *NN'*-methylenebisacrylamide. These gels did not contain SDS and were soluble in 2% (w/v) periodic acid (Anker, 1970). After electrophoresis the gels were stained in 0.25% (w/v) Coomassie G-250 for 4h. The gels were destained in 7% (v/v) acetic acid containing 10% (v/v) methanol.

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

Results

In preliminary studies, β-bungarotoxin was iodinated by a modification of the method of Marchalonis (1969) using lactoperoxidase, but it was found that the ^{125}I-labelled β-bungarotoxin (sp. radioactivity 13.6Ci/mmol) has no neurotoxic activity. This was tested by intravenous injection into mice and by application to a nerve–muscle preparation *in vitro*.

The labelling procedure used here was a modification of the method of Churchich (1965), which involved the formation of an imine between the aldehyde on pyridoxal 5'-phosphate and the primary amino group on an available basic amino acid (probably lysine) of the polypeptide chain of β-bungarotoxin. This unstable Schiff base was reduced with NaB^3H_4 to the more stable secondary amine. Fig. 1 shows the emergence of [^{3}H]pyridoxylated β-bungarotoxin from the column of CM-Sephadex C-25. There was a single peak of protein, which coincided with the peak of radioactivity. It was shown on a similar column that, at pH7.0, unlabelled β-bungarotoxin emerges from CM-Sephadex C-25 at an ionic concentration of about 900mM, unlike the [^{3}H]pyridoxylated β-bungarotoxin that emerges at 600mM. Inspection of Fig. 1 shows that there was no detectable second peak of protein at an ionic concentration of 900mM.

The specific radioactivity of the [^{3}H]pyridoxylated β-bungarotoxin varied from batch to batch between 1.47 and 8.41 Ci/mmol. Precipitation of the labelled toxin, in the presence of 10mg of bovine serum albumin/ml, with 5% (w/v) trichloroacetic acid showed that over 96% of the radioactivity was removed from solution. The biological activity of the [^{3}H]pyridoxylated β-bungarotoxin was measured by intravenous injection into the tail vein of mice. It had an LD_{50} about 10 times greater than that of the unlabelled toxin (0.3 as compared with 0.01–0.03 μg/g body wt.). Death occurred after the characteristic toxic effects of β-bungarotoxin (Chang & Lee, 1963) had been observed. In addition, the biological activity of [^{3}H]pyridoxylated β-bungarotoxin was tested by its effect on a nerve–muscle preparation *in vitro* and was found to block neuromuscular transmission. A transient increase in the rate of generation of miniature end-plate potentials was observed, similar to the effect of non-radioactive β-bungarotoxin. There was also a decrease in the evoked release of synaptic transmitter, measured indirectly by the contractile force of isometric twitch responses to single nerve stimuli (MacDermot *et al.*, 1978), as observed when using non-radioactive toxin, but the time course of this effect with [^{3}H]pyridoxylated β-bungarotoxin was altered (and in general was much slower).

Pilot kinetic studies were carried out for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes in the absence of bovine serum albumin. Under these conditions, the binding of [^{3}H]pyridoxylated β-bungarotoxin was not saturable, but increased in a linear fashion with toxin concentration through three orders of magnitude. It was then shown that bovine serum albumin at concentrations greater than 20mg/ml prevented the binding of [^{3}H]pyridoxylated β-bungarotoxin to plastic, glass and other surfaces. For this reason bovine serum albumin was added to the incubations at a concentration of 30mg/ml and thereafter saturable binding to the synaptosomes was detected.

To establish a time curve for the binding of [^{3}H]-pyridoxylated β-bungarotoxin to synaptosomes, suspensions of synaptosomes were incubated with 0.5 μM-[^{3}H]pyridoxylated β-bungarotoxin. At selected times, the reaction was stopped as described under 'Methods', except that the synaptosomes were washed as quickly as possible and only four times. The reaction time was measured from the moment that the [^{3}H]pyridoxylated β-bungarotoxin was added to the end of the first centrifugation. The amount of β-bungarotoxin bound per mg of synaptosomal protein was plotted as a function of time (min) and is shown in Fig. 2. Half-maximal binding occurred under these conditions in 3min and 30s. A similar study (results not shown) showed that once 95%

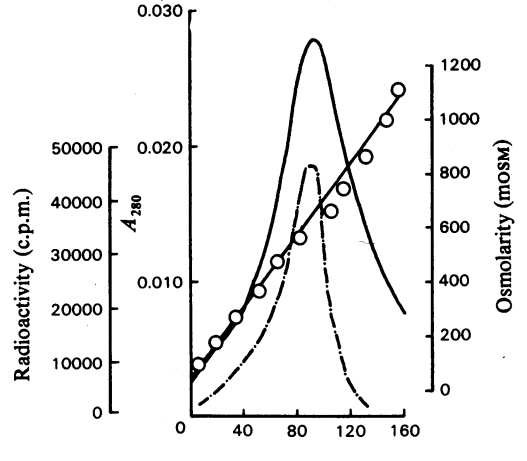

Fig. 1. *Chromatography of* [^{3}H] *pyridoxylated β-bungarotoxin*

Ion-exchange chromatography of [^{3}H]pyridoxylated β-bungarotoxin on CM-Sephadex C-25. The protein was eluted from the column with a NaCl gradient (○, 50mM to 1.0M) in 5mM-sodium phosphate buffer, pH7.0. The peak of protein (——) is coincident with the peak of radioactivity (-·-·-·).

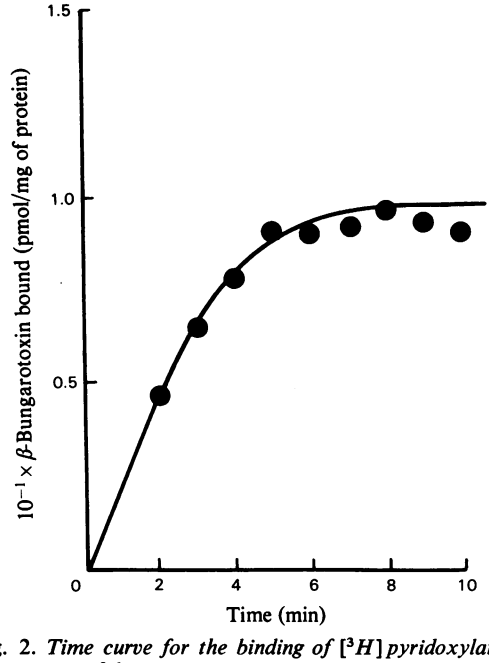

Fig. 2. *Time curve for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes*
Incubations (200 μl) contained a synaptosomal suspension in 5 mM-sodium phosphate buffer, pH 7.0, 150 mM-NaCl, 0.5 μM-[^{3}H]pyridoxylated β-bungarotoxin and 30 mg of bovine serum albumin/ml. Additional experimental details are provided in the text.

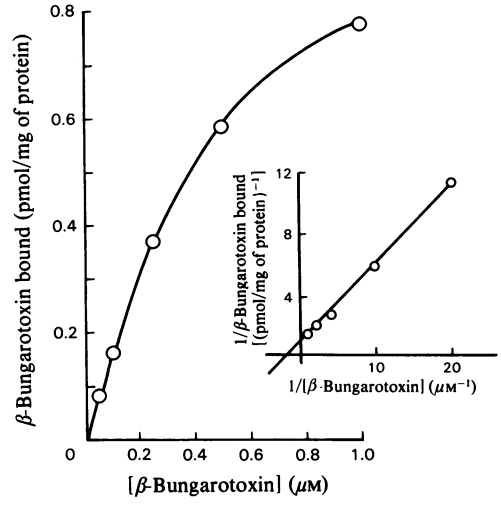

Fig. 3. *Concentration curve for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes*
Incubations (200 μl) contained a synaptosomal suspension in 5 mM-sodium phosphate buffer (pH 7.0)/150 mM-NaCl, [^{3}H]pyridoxylated β-bungarotoxin at concentrations of 50 nM to 1 μM and 30 mg of bovine serum albumin/ml. The reactions were terminated after 1 h. Additional experimental details are provided in the text.

saturation was reached under these conditions, the amount of [^{3}H]pyridoxylated β-bungarotoxin bound remained constant for at least 1.5 h.

To establish a concentration curve for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes, suspensions of synaptosomes were incubated with ^{3}H-pyridoxylated β-bungarotoxin at concentrations of 50 nM to 1 μM. The incubations were allowed to proceed for 1 h and were then terminated as described under 'Methods'. Fig. 3 shows the concentration curve and the inset is a double-reciprocal plot of the same data (Lineweaver & Burk, 1934). The K_a (app.) was 0.83 μM. The receptor concentration was calculated from the ordinate intercept and was 1.43 pmol/mg of synaptosomal protein. Fig. 4. shows a Hill (1910) plot. It presents an analysis of the same data and the Hill coefficient (h) equals 1.0, hence there is no co-operativity of binding. Fig. 5 shows a Scatchard (1949) plot and it can be seen that there is a single slope and thus only one class of receptor. The K_d (app.) was calculated from the slope as 0.81 μM.

The finding of Oberg & Kelly (1976) that the bind-

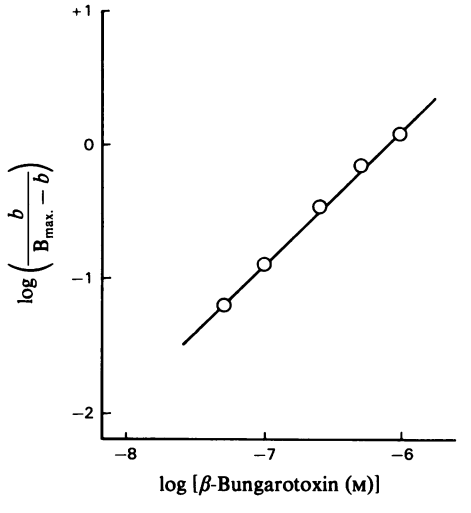

Fig. 4. *Hill plot for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes*
Experimental details are given in the legend to Fig. 3. In this plot, b is the amount of toxin bound in the reaction (pmol of [^{3}H]pyridoxylated β-bungarotoxin bound/mg of protein) at a particular concentration of β-bungarotoxin. $B_{max.}$ is the maximum amount of toxin bound and was determined from Fig. 3.

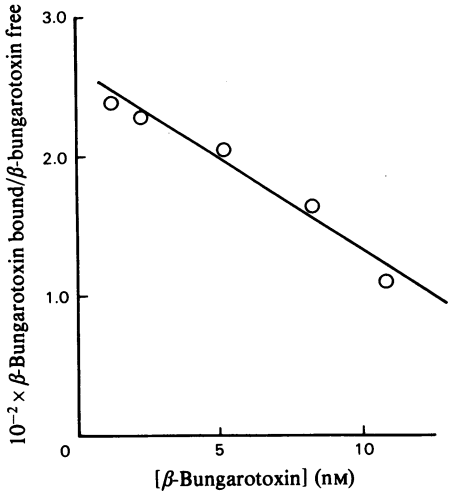

Fig. 5. *Scatchard plot for the binding of [^{3}H] pyridoxylated β-bungarotoxin to synaptosomes*
Experimental details are given in the legend to Fig. 3. Concentration of toxin (nM) is on the abscissa. The ordinate shows the quotient of mol of toxin bound over the mol of free toxin in each 200 μl of incubation medium.

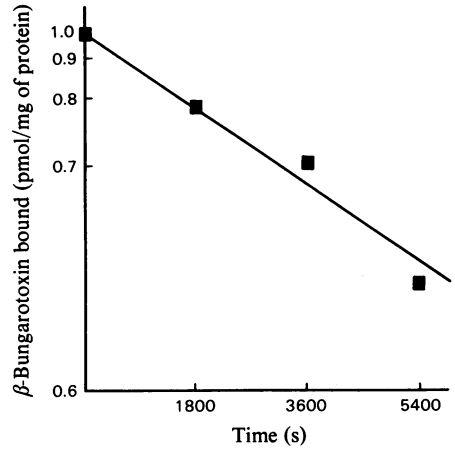

Fig. 6. *Dissociation of the complex of [^{3}H] pyridoxylated β-bungarotoxin and its receptor*
Semilogarithmic plot of the dissociation of the complex as a function of time. Incubation media (200 μl) contained 9.85 nM-complex in 5 mM-sodium phosphate buffer, pH 7.0, 150 mM-NaCl, 0.5 μM-non-radioactive pyridoxal 5′-phosphate-labelled β-bungarotoxin and 30 mg of bovine serum albumin/ml. Additional experimental details are provided in the text.

ing of β-bungarotoxin was unaffected by the concentration of Ca^{2+} (0–2.5 mM) or Sr^{2+} ions (0–5 mM) was confirmed. The addition of EGTA (0–0.2 mM) had no effect either.

The true dissociation constant (K_d) was determined by the method of Chang *et al.* (1975). The K_d (app.) was determined for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes at various dilutions of the receptor. By extrapolation, the K_d (app.) at an infinite dilution of receptor may be determined, which equals the true K_d. This was 0.37 μM.

The dissociation constant (K_d) for the binding of [^{3}H]pyridoxylated β-bungarotoxin to synaptosomes was also determined by measuring the rate constants for the forward and back reactions. The rate constant for the forward reaction (k_{+1}) was calculated from the data in Fig. 1. The molar concentration of receptor was calculated from the receptor density in synaptosomes. The value of k_{+1} was $4.92 \times 10^2 M^{-1} \cdot s^{-1}$. The rate constant of the back reaction (k_{-1}) was determined from the rate of dissociation of the complex of [^{3}H]pyridoxylated β-bungarotoxin and its receptor in synaptosomes. Incubations of synaptosomes in 0.5 μM-[^{3}H]pyridoxylated β-bungarotoxin were prepared as described under 'Methods'. After an incubation of 1 h the synaptosomes were washed six times as above. The washed pellets were finally resuspended in 1 ml of 5 mM-sodium phosphate buffer, pH 7.0, containing 150 mM-NaCl and 30 mg of bovine serum albumin/ml. To these incubations of synaptosomes with bound [^{3}H]pyridoxylated β-bungarotoxin was then added 'non-radioactive pyridoxal 5′-phosphate-labelled β-bungarotoxin' (see under 'Methods') to a final concentration of 0.5 μM. The initial concentration of the bound [^{3}H]-pyridoxylated β-bungarotoxin was calculated from the radioactive counts on the suspended synaptosomes and was 9.9 nM. The incubations were then allowed to proceed for zero, 30, 60 and 90 min and the radioactivity still bound to the tissue fragments was determined as described under 'Methods'. Fig. 6 shows the semilogarithmic plot for the dissociation of [^{3}H]pyridoxylated β-bungarotoxin from its receptor. From these data the rate constant (k_{-1}) was calculated to be $1.01 \times 10^{-4} s^{-1}$. The dissociation constant (K_d) was derived from the expression k_{-1}/k_{+1}, whence K_d equals 0.21 μM.

The dissociation constant for the binding of unlabelled β-bungarotoxin to synaptosomes was determined by the method of Dixon (1953). The unlabelled toxin showed competitive inhibition of the reaction of [^{3}H]pyridoxylated β-bungarotoxin and its receptor. The K_i was 25 nM. It follows that the labelling procedure decreases the affinity of β-bungarotoxin for its receptor. It was for this reason that 'non-radioactive pyridoxal 5′-phosphate-labelled

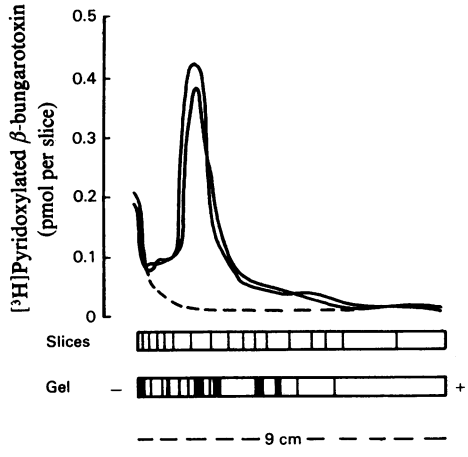

Fig. 7. *A protein receptor of [3H]pyridoxylated β-bungarotoxin isolated from rat brain*

Soluble proteins were extracted from rat brain by homogenizing in 320mM-sucrose, followed by ultrasonication and centrifugation at 100000*g* ($r_{av.}$ 8.5cm) for 1h. The supernatant was incubated with 0.5μM-[3H]pyridoxylated β-bungarotoxin and thereafter 50μl volumes of this incubation were applied to 8% (w/v) polyacrylamide gels. Electrophoresis was carried out at pH8.3 towards the anode. The location of the protein bands after electrophoresis is shown. In addition, the location of a radioactive peak is shown in duplicate gels (——). In a control gel, [3H]pyridoxylated β-bungarotoxin alone was applied. A corresponding peak of radioactivity was not seen in this gel (– – – –). The radioactive peak coincided with a protein band located in slice 7 of the gel from the cathode end. Additional experimental details are provided in the text.

β-bungarotoxin' was used in excess during the back reaction, rather than unlabelled β-bungarotoxin.

Isolation of β-bungarotoxin receptor

The β-bungarotoxin receptor was isolated from rat brain by using [3H]pyridoxylated β-bungarotoxin. The cerebral cortex of a 520g male rat was homogenized with an Ultra-Turrax tissue homogenizer (Janke & Kunel, Staufen i Breisgau, Germany) in 20ml of 320mM-sucrose at 4°C. This was then ultrasonicated in 5s bursts for a total of 25s at 4°C in a N_2 atmosphere and centrifuged at 100000*g* ($r_{av.}$ 8.5cm) for 1h at 4°C. The protein concentration of the supernatant was 3.5mg/ml. An incubation was performed with 400μl of supernatant containing 0.5μM-[3H]pyridoxylated β-bungarotoxin. The incubation was left for 1h at room temperature. A control incubation was also performed with 400μl of 320mM-sucrose containing the same concentration of [3H]pyridoxylated β-bungarotoxin. After 1h, 10μl of Bromophenol Blue marker dye was added to each incubation, which was then layered in 50μl volumes on to the upper surface of 8% (w/v) polyacrylamide gels, containing *NN'*-diallyltartardiamide (see under 'Methods'). The electrode solution was 50mM-Tris/HCl buffer, pH8.3, and the samples were run in the gels towards the anode at 6mA/tube for 5h at room temperature.

At the end of the electrophoretic run, the gels were stained as described under 'Methods' and two of those showing the separated brain proteins were sliced as shown in Fig. 7. Each slice was dissolved in 1ml of 2% (w/v) periodic acid (about 2h) and each clear solution was then added to 10ml of scintillant and the individual samples were counted for radioactivity in a scintillation counter for a time sufficient to give 10^4 counts. Fig. 7 shows the location of the proteins within the gel and the occurrence of a single peak of radioactivity over one particular band (in slice 7 from the cathode end). In addition the radioactivity is shown in the slices of gel taken from the control (i.e. [3H]pyridoxylated β-bungarotoxin alone). It is clear that at pH8.3, the toxin does not migrate towards the anode, but in the electrophoretic separation of the brain proteins, the [3H]pyridoxylated β-bungarotoxin has migrated into the gel with its receptor as a complex whose isoelectric point is below 8.3.

Fig. 7 shows that this complex is located in slice 7 from the cathode end of the gel. Two additional gels were taken and sliced as above. The two pieces corresponding to slice 7 were then cut into small pieces and placed in a single glass tube. To this, 1ml of 0.1% (w/v) SDS containing 0.1mM-phenylmethanesulphonyl fluoride was added. The protein was then extracted from the gel pieces by heating to 100°C for 5min. The supernatant (S1) was pipetted off. The procedure was then repeated giving another 1ml supernatant (S2). The two supernatants were pooled and then concentrated by Amicon filtration through a PM-10 filter to a final volume of 100μl. This was heated with 5μl of 10% (v/v) 2-mercaptoethanol at 100°C in a water bath.

In a preliminary molecular-weight determination by the method of Weber & Osborn (1969), the extracted receptor had a mol.wt. of about 200000. The electrophoretic mobility under these conditions was then compared with that of myosin. Three 8% (w/v) polyacrylamide gels containing *NN'*-methylenebisacrylamide (see under 'Methods') were prepared and samples for electrophoresis were made by the method of Weber & Osborn (1969). The electrode solution contained 50mM-Tris/HCl buffer, pH8.3, containing 0.1% (w/v) SDS. The gels were run for 5h at 6mA/tube at room temperature. Fig. 8 shows densitometer scans of the gels. The myosin light chains, which represent about 10–12% of the myosin molecule, were not clearly visible and they cannot be seen on the scan. In subsequent studies, with greater amounts of the myosin preparation, the light chains were identified.

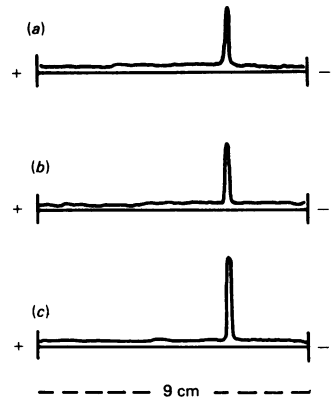

Fig. 8. *Electrophoresis of the β-bungarotoxin receptor and myosin on polyacrylamide gels*

Chromoscan densitometer scans of polyacrylamide gels after electrophoresis with SDS under reducing conditions of: (*a*) myosin, (*b*) β-bungarotoxin receptor, and (*c*) a mixture of myosin and the β-bungarotoxin receptor. The electrophoresis was performed at pH8.3 and the gels were run for 5h at 6mA/tube at room temperature. Additional experimental details are provided in the text.

Discussion

β-Bungarotoxin has been labelled with pyridoxal 5′-phosphate by the method of Churchich (1965). The unstable Schiff base formed was reduced with NaB^3H_4 to the more stable secondary amine. For optimum labelling conditions, it was found that the excess pyridoxal 5′-phosphate should not be separated from the Schiff base before reduction as suggested by Cooper & Reich (1972), since this technique yielded large amounts of unlabelled toxin. Under conditions described in the text, no unlabelled β-bungarotoxin was detected after reduction. Adequate NaB^3H_4 had to be added, however, to reduce both the Schiff base and the excess pyridoxal 5′-phosphate.

Iodination of β-bungarotoxin using lactoperoxidase appeared to render it non-toxic; however, Oberg & Kelly (1976) reported that β-bungarotoxin may be iodinated by using chloramine-T. The labelled toxin was not tested, however, for its neurotoxic properties, but only for its putative phospholipase A activity. Labelling with pyridoxal 5′-phosphate did not render the β-bungarotoxin non-toxic, but did decrease its affinity of binding to synaptosomes [K_d (app.) was decreased from 25nM to 0.83μM].

The true dissociation constant (K_d) for the binding of [^{3}H]pyridoxylated β-bungarotoxin to its receptor was determined as 0.37μM by the method of Chang *et al.* (1975). This compared well with the K_d determined from the rate constants of the forward and back reactions. The value of k_{-1}/k_{+1} was 0.21μM. In published work to date, two values for the dissociation constant have been given, one for labelled and the other for unlabelled toxin. Oberg & Kelly (1976) studying the binding of ^{125}I-labelled β-bungarotoxin to a membrane preparation of brain, determined the K_d to be 1.7nM, whereas Sen *et al.* (1976), studying the inhibition of choline uptake into synaptosomes produced by unlabelled β-bungarotoxin, found that the K_i of β-bungarotoxin was 50nM. Our equivalent value of K_i for the unlabelled toxin is 25nM, which is in fairly good agreement.

Analysis of the binding of [^{3}H]pyridoxylated β-bungarotoxin with Hill and Scatchard plots suggested respectively that there was no co-operativity of binding and only a single class of receptor sites with a bimolecular association of [^{3}H]pyridoxylated β-bungarotoxin and its receptor. Comparison of the relative concentrations of receptor in various subcellular fractions of rat brain shows that it is distributed in the pmol/mg of protein range. This agrees with the results of Oberg & Kelly (1976), who found the greatest receptor concentration to be in brain (13pmol/mg of protein) with relatively less in liver and erythrocytes. The possibility that the β-bungarotoxin receptor might be myosin was first considered when it was shown that the receptor was distributed widely in several membrane preparations from brain, but that the concentration was greatest in the membrane of the synaptic vesicles. Earlier work showed that an actin-like protein and tropomyosin could be extracted from chick brain (Fine & Bray, 1971; Fine *et al.*, 1973) and subsequent analysis of the different proteins present in various membrane preparations from brain demonstrated the presence of myosin (Blitz & Fine, 1974). They also found that the greatest concentration of myosin was within the membrane of synaptic vesicles and the greatest concentration of actin was within the plasma membrane of nerve terminals.

If the suggestion of Chang *et al.* (1973), that β-bungarotoxin might bind to specific macromolecule(s) involved in transmitter release, were correct, then the possibility that the receptor is myosin would accord with their hypothesis. Berl *et al.* (1973) have proposed a model for the release of synaptic vesicles, which involves the interaction of stenin (a myosin-like protein) and neurin (an actin-like protein) located in the membrane of synaptic vesicles and the plasma membrane respectively. Fusion of the synaptic vesicle and the plasma membrane, it is proposed, is dependent on a Ca^{2+}-dependent adenosine triphosphatase located in the actomyosin filaments.

β-Bungarotoxin does not cross the plasma membrane of the nerve terminal (Howard & Wu, 1976; Oberg & Kelly, 1976). Thus if β-bungarotoxin were to bind to myosin it is clear that at least a portion of

the myosin molecule would have to span the plasma membrane and be available for toxin binding on the outer surface of the cell. This structural orientation of membrane myosin has been demonstrated by Willingham *et al.* (1974).

Finally, the demonstration that putative protein receptor of β-bungarotoxin co-migrates with muscle myosin on polyacrylamide gel is clearly not sufficient evidence on its own to identify it as myosin with certainty. Further, the myosin of brain can be distinguished from that in other tissues (Burridge & Bray, 1975). The possibility that the receptor may be clathrin (Pearse, 1975) has also been considered, since it has a similar molecular weight and is thought to be involved in vesicle membrane movements. Although its function is less clear, there is a third protein with similar molecular weight (LETS, i.e. large, external, transformation-sensitive) that appears related to areas of cell–cell contact (Chen, 1977).

In the same molecular-weight range a protein has been isolated from smooth-muscle cells and has been termed filamin. It has a similar association to cellular filaments, as does myosin, but it is distinguishable from myosin (Wang *et al.*, 1975). Further work is required to distinguish these various possible receptor proteins for β-bungarotoxin.

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