Interaction of Di-iodinated ^{125}I -labelled α -Bungarotoxin and Reversible Cholinergic Ligands with Intact Synaptic Acetylcholine Receptors on Isolated Skeletal-Muscle Fibres from the Rat

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1. Intact synaptic acetylcholine receptors on freshly isolated rat skeletal-muscle fibres were characterized by their interaction with di-iodinated ^{125}I -labelled α -bungarotoxin, acetylcholine and other cholinergic ligands at room temperature (22°C). 2. The time course and concentration dependence of ^{125}I -labelled α -bungarotoxin association conformed to a bimolecular mechanism. In time-course experiments with different concentrations of ^{125}I -labelled α -bungarotoxin (1.4–200 nM) the bimolecular-association rate constant, k_{+1} , was $(2.27 \pm 0.49) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ s.d., $n = 10$). In concentration-dependence experiments, k_{+1} was $2.10 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.74 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ with 10 and 135 min incubations respectively. In association experiments the first-order rate constant was proportional to the ^{125}I -labelled α -bungarotoxin concentration. ^{125}I -Labelled α -bungarotoxin dissociation was first order with a dissociation constant, k_{-1} , less than or equal to $3 \times 10^{-6} \text{ s}^{-1}$ (half-life $\geq 60 \text{ h}$.) The results indicated a single class of high-affinity toxin-binding sites at the end-plate with an equilibrium dissociation constant, K_d , equal to or less than 100 pM. The number of toxin-binding sites was $(3.62 \pm 0.46) \times 10^7$ (mean $\pm$ s.d., $n = 22$) per rat end-plate. 3. The apparent inhibitor dissociation constants, K_i , for reversible cholinergic ligands were determined by studying their effect at equilibrium on the rate of ^{125}I -labelled α -bungarotoxin binding. There was heterogeneity of binding sites for cholinergic ligands, which were independent and non-interacting with antagonists. In contrast agonist affinity decreased with increasing receptor occupancy. Cholinergic ligands in excess inhibited over 90% of ^{125}I -labelled α -bungarotoxin binding. 4. Cholinergic ligand binding was accompanied by an increase in entropy, which was greater for the agonist carbachol ($\Delta S^\circ = +0.46 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) than the antagonist tubocurarine ($\Delta S^\circ = +0.26 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). 5. The entropy and affinity changes that accompanied agonist binding suggested that agonists induced significant conformational changes in intact acetylcholine receptors. 6. The affinity and specificity of ^{125}I -labelled α -bungarotoxin and tubocurarine binding to synaptic acetylcholine receptors from slow and fast muscle fibres were the same. 7. The study of binding only requires milligram amounts of tissue and may have application to other neurobiological studies and to the study of human neuromuscular disorders.

Factors controlling the expression of acetylcholine receptors in their membrane environment have been extensively studied by physiological and biochemical methods (for reviews see Rang, 1975; Neumann & Bernhardt, 1977; Heidmann & Changeux, 1978). Both approaches have shown that the number, distribution and conformational state of these receptors can be affected acutely by pharmacologically active agents (Franklin & Potter, 1972; Changeux *et al.*, 1976; Sugiyama *et al.*, 1976) and chronically by changes in neuronal and developmental influences (Almon *et al.*, 1974; Brockes & Hall, 1975*a,b*).

Important to the understanding of these changes and to the role of acetylcholine receptors in neuromuscular transmission is the normal molecular conformation of these receptors *in vivo* and their interaction with acetylcholine and other cholinergic ligands.

The discovery that certain snake neurotoxins, including α -bungarotoxin, bind with high affinity to acetylcholine receptors opened the way for their isolation and detailed biochemical analysis. Because acetylcholine receptors are only present in small quantities in normal intact tissue, they have been characterized kinetically after extraction either from the rich source of fish electric organs (for review see Heidmann & Changeux, 1978) or more recently

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from denervated and normal mammalian muscle (Brookes & Hall, 1975a,b; Colquhoun & Rang, 1976; Almon & Appel, 1976a,b). Studies on detergent-solubilized- and homogenized-membrane preparations have given widely differing kinetic values with both a heterogeneity (Eldefrawi & Eldefrawi, 1973; Brookes & Hall, 1975a,b; Raftery *et al.*, 1976; Bulger *et al.*, 1977; Maelicke *et al.*, 1977) and a homogeneity (Weber & Changeux, 1974; Colquhoun & Rang, 1976; Weiland *et al.*, 1976) of toxin-binding sites being reported. Whether these results truly reflect receptor characteristics within their membrane environment remains uncertain, as detergents and homogenization are known to alter both the affinity and specificity of ligand binding (Franklin & Potter, 1972; Changeux *et al.*, 1976; Popot *et al.*, 1976).

Previous studies of toxin binding to intact acetylcholine receptors in whole muscle tissue (Miledi & Potter, 1971; Berg *et al.*, 1972; Porter *et al.*, 1973; Chiu *et al.*, 1974; Libelius *et al.*, 1975) have suggested that there may be more than one type of acetylcholine receptor at the intact synapse, but slow and variable diffusion of ligands in such preparations (Miledi & Potter, 1971) complicates the determination of kinetic constants.

In an attempt to approximate to the normal receptor configuration *in vivo* and to overcome the problems of diffusion, the interaction of ^{125}I -labelled α -bungarotoxin, acetylcholine and other cholinergic ligands with intact synaptic acetylcholine receptors on freshly isolated skeletal-muscle fibres in the rat has been studied.

Materials and Methods

Materials

CM-Sephadex C-25 and Sephadex G-10 were from Pharmacia Fine Chemicals, Uppsala, Sweden, Na^{125}I (sp. radioactivity 1750 Ci/mmol) and gamma counter checking source (^{129}I) were from The Radiochemical Centre, Amersham, Bucks., U.K., freeze-dried venom of *Bungarus multicinctus*, ATP, bovine serum albumin (RIA grade), carbamoylcholine chloride and eserine sulphate were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K., bovine serum albumin (Cohn fraction 5) and acetylthiocholine were from Koch-Light, Colnbrook, Bucks., U.K., atropine sulphate was from Antigen, Roscrea, Ireland, *d*-tubocurarine chloride was from The Wellcome Foundation, Berkhamsted, Herts., U.K., sodium pentobarbitone was from May and Baker, Dagenham, Essex, U.K., Whatman 3MM chromatographic paper was from Whatman, Maidstone, Kent, U.K., 2.5% (v/v) Viokase solution was from Grand Island Biological, Grand Island, NY, U.S.A., butan-1-ol for chromatography was from BDH Chemicals, Poole, Dorset, U.K., Ilford K2 nuclear emulsion was from Ilford Ltd., Ilford, Essex, U.K., 3-iodo-L-tyrosine and

3,5-di-iodo-L-tyrosine were from Aldrich Chemical Co., Gillingham, Dorset, U.K., glucose oxidase was from Worthington Corp., Freehold, NJ, U.S.A., and lactoperoxidase was from Calbiochem, Bishops Cleeve, Herts., U.K. All other reagents were of analytical grade.

Preparative methods

Preparation of toxin for experiments. α -Bungarotoxin. This was separated from the crude venom of *B. multicinctus* by ion-exchange column chromatography (CM-Sephadex C-25) as previously described (Macdermot *et al.*, 1978). To check its purity, the α -bungarotoxin was twice re-chromatographed, in identical fashion, and on both occasions a single symmetrical protein peak was eluted. Finally, the α -bungarotoxin was concentrated in 500 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.0, by further ion-exchange chromatography and stored in portions at -30°C .

^{125}I -labelled α -bungarotoxin. This was prepared in a fume cupboard at room temperature (22°C) with a standardized reaction procedure as follows: 75 μl of D-glucose (0.5 mg/ml), 40 μl of glucose oxidase (50 $\mu\text{g}/\text{ml}$) and 40 μl of lactoperoxidase (100 $\mu\text{g}/\text{ml}$) were added in that order to 75 μg of α -bungarotoxin in 150 μl of 500 mM-NaCl/100 mM-sodium phosphate buffer, pH 7.0. Na^{125}I (5 mCi) was then added, followed 3 min later by 34 μl of NaI (75 $\mu\text{g}/\text{ml}$). After 12 min the reaction was completed by addition of an equal volume (390 μl) of 200 mM-NaI/3 mM-sodium phosphate buffer, pH 7.0. To separate the unreacted iodide, the reaction mixture was immediately applied to a Sephadex G-10 column (20 cm $\times$ 1.5 cm) pre-equilibrated in a solution of 5 mg of bovine serum albumin/ml of 3 mM-phosphate buffer, final pH 7.3, and eluted with the same buffer in 1 ml fractions at a rate of 1 ml/min. The first peak (i.e. ^{125}I -labelled α -bungarotoxin) contained over 90% of the applied radioactivity, which suggested that the labelled toxin was di-iodinated ^{125}I -labelled α -bungarotoxin as the molar ratio of total iodide to toxin in the reaction mixture was 2:1. Subsequently the ^{125}I -labelled α -bungarotoxin was adsorbed on a CM-Sephadex C-25 column (0.9 cm $\times$ 3 cm) pre-equilibrated in a solution of 5 mg of bovine serum albumin/ml of 3 mM-phosphate buffer, final pH 7.3, washed with 25 ml of this same buffer to remove the enzyme, electrostatically bound iodide and damaged radioactive protein fragments and then eluted in 1 ml fractions at a rate of 1 ml/min with a solution of 5 mg of bovine serum albumin/ml of 150 mM-sodium chloride/3 mM-sodium phosphate buffer, final pH 7.3. The ^{125}I -labelled α -bungarotoxin protein peak from this column was pooled, gamma-counted to determine the specific radioactivity (usually approx. 400 Ci/mmol) and then stored in portions at -70°C . Each batch of radioactively labelled toxin was used for

a maximum of 8 weeks. The use of plastic tubes and pipettes and the immediate addition of an excess of bovine serum albumin after the iodination reaction prevented the non-specific adsorption and loss of radioactively labelled toxin.

Characterization of ^{125}I -labelled α -bungarotoxin. Characterization by ion-exchange column chromatography (see e.g. Vogel *et al.*, 1972; Colquhoun & Rang, 1976) and of hydrolysed ^{125}I -labelled α -bungarotoxin by paper chromatography (Vogel *et al.*, 1972) confirmed that 90% of the ^{125}I -labelled α -bungarotoxin used in these experiments was diiodinated.

Biological toxicity and specificity. Intraperitoneal injection (0.3 $\mu\text{g/g}$ of rat) of radioactively labelled and unlabelled toxins resulted in lethal respiratory failure and decreased the amplitude of the foot-pad compound-muscle action potential evoked by supra-maximal sciatic-nerve stimulation. In nerve-muscle preparations, by methods previously described (Macdermot *et al.*, 1978), radioactively labelled and unlabelled toxins abolished miniature end-plate potentials, and the isometric twitch tension evoked by nerve stimulation, but not that evoked by direct muscle stimulation. Radioautographs showed high end-plate and low non-end-plate 'grain' counts (see Fig. 6).

Experimental methods

For each experiment a Sprague-Dawley rat (180–250 g) was killed with an intraperitoneal injection of sodium pentobarbitone (0.5 mg/g). The lateral part of the lateral head of the gastrocnemius muscle and the soleus muscle were immediately removed into a medium consisting of 50% glycerol (v/v) in solution A (solution A contained 131 mM- Na^+ , 5 mM- K^+ , 2 mM- Ca^{2+} , 111 mM- Cl^- , 29 mM-lactate, 2 mM-phosphate and 5 mg of bovine serum albumin/ml, final pH 7.3). Single fibres and small bundles containing two to ten muscle fibres (approx. 1.5 cm long) were teased out intact under a dissecting microscope, washed in solution A to remove the glycerol and incubated in ^{125}I -labelled α -bungarotoxin plus or minus ligand in solution A at room temperature (22°C). The incubations were carried out in sterile plastic culture dishes (diameter 15 mm) with a constant reaction volume for each experiment of 1 ml or 2 ml. The fibres were then washed rapidly in 4 $\times$ 5 ml of solution A, fixed in 2.5% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.3, and the end-plates were identified by the method of Karnovsky & Roots (1964) for acetylcholinesterase. Each fibre was divided into end-plate and contiguous non-end-plate segments of approximately equal length (300 μm). Equal numbers of end-plate and non-end-plate segments were counted in a gamma counter. The specific binding to acetylcholine receptors was taken to be the difference in gamma counts between

the end-plate and non-end-plate segments and expressed in amol bound/end-plate. Only intact fibres with end-plates directly exposed to the incubation medium were counted. In preliminary studies the rate of toxin binding to different-sized muscle bundles was uniform for bundles containing up to 20 fibres. However, when whole blocks of muscle tissue were incubated in radioactively labelled toxin, the rate of toxin binding to deeper fibres was markedly decreased.

The number of fibres in each incubation (20–150), the number of incubations in each experiment (4–10), and the gamma-counting conditions were chosen so that the 95/100 proportional gamma-counting error of estimation was always less than 5%. A LKB-Wallac 80000 Gamma counter was used for counting radioactivity. The counting efficiency, determined by a ^{129}I -gamma checking source (simulating 0.08 μCi of ^{125}I), was 55%. Before gamma counting, all pots were checked to make sure the background was less than 20 c.p.m.

In some experiments a small portion of each fibre was histochemically typed with the myosin ATPase reaction (Brooke & Kaiser, 1970) before fixation and the remaining segment (containing the end-plate) was fixed and prepared for radioautography. For this purpose the fibres were mounted on specially cleaned glass slides and dipped in 24 ml of Ilford K2 nuclear emulsion diluted with 6 ml of water and 0.3 ml of glycerol at 43°C in a dark room. They were dried and stored in light-proof boxes at 4°C for various periods of time and then they were developed. In this way the binding characteristics of fibres of known histochemical type were studied.

Analysis of Binding Data

Binding of toxin

The binding of toxin (T) to acetylcholine receptors (R) was studied under pseudo-first-order conditions and analysed by using the reaction schemes:

$$k = k_{+1}[\text{T}] \quad (1a)$$

where k and k_{+1} are the first-order and bimolecular-second-order association-rate constants respectively, k_{-1} is the dissociation-rate constant and $K_d = k_{-1}/k_{+1}$, the equilibrium dissociation constant.

Binding of reversible cholinergic ligands

This was studied by their effect at equilibrium on the rate of toxin binding. Provided that the rate of toxin binding is much slower than the rate of ligand equilibration, then according to theory (see Colquhoun & Rang, 1976) the rate constants for toxin

binding in the presence ($k_{\text{obs.}}$) and the absence (k) of the ligand will be related as follows:

$$k_{\text{obs.}} = \frac{k}{1 + ([I]/K_i)^h} \quad (2)$$

where $[I]$ and K_i are the concentration and an apparent inhibitor dissociation constant of the ligand respectively and h is the Hill coefficient.

If r is defined as the ratio of these rate constants (i.e. $r = k/k_{\text{obs.}}$) then:

$$r - 1 = ([I]/K_i)^h \quad (2a)$$

A double-logarithmic plot of $(r-1)$ against $[I]$ is formally similar to a Schild (1947) or a Hill (1910) plot and will have a slope of h , the Hill coefficient. Also $[I] = K_i$ when $r-1 = 1$ (i.e. when $r = 2$ and there is 50% inhibition of toxin binding), which will give a better estimate of K_i than extrapolation beyond the data to the intercept.

Also substitution of eqn. (1a) into eqn. (2) gives:

$$k_{\text{obs.}} = \frac{k_{+1}[T]}{1 + ([I]/K_i)^h} \quad (2b)$$

The addition of a purely competitive inhibitor in fixed concentration, $[I]$, would decrease the slope of a plot of $k_{\text{obs.}}$ against different toxin concentrations, $[T]$. The plots, however, would remain linear.

Binding of unlabelled toxin

If, here, r is defined as the ratio of labelled toxin binding in the absence and presence of unlabelled toxin (T^*) concentration, then at saturation:

$$r - 1 = ([T^*]/[T]) \quad (3)$$

In this case a double-logarithmic plot of $(r-1)$ against the concentration of unlabelled toxin ($[T^*]$) will be linear and of unit slope provided that the radioactively labelled and unlabelled toxins bind competitively to the same site. Of course in this case the slope, although in some ways comparable, is not the Hill coefficient.

Unless otherwise indicated, all the slopes and kinetic values quoted in the present paper were derived by linear least-squares regression analysis of the experimental data. Where appropriate the correlation coefficient, r , is given in the Figure legends.

Results

Time course of ^{125}I -labelled α -bungarotoxin association

The binding of radioactively labelled toxin to the non-end-plate regions increased linearly with time and showed no evidence of saturation (Figs. 1a and 1b). In other experiments it was shown that non-end-plate binding was unaffected by cholinergic ligands or unlabelled toxin. Non-end-plate binding

was therefore taken to be 'non-specific' and subtracted from the total binding at the end-plate region to obtain the specific binding to synaptic acetylcholine receptors.

Specific end-plate binding with ^{125}I -labelled 200 nm- α -bungarotoxin saturated in 10 min (Fig. 1a).

Fig. 1. Time course of ^{125}I -labelled α -bungarotoxin association

Fibres were incubated in ^{125}I -labelled 200 nm- (a) and 54 nm- (b) α -bungarotoxin. The specific binding to end-plates ($\bullet$) was taken to be the difference between the total ($\circ$) and non-end-plate binding ($\square$). (c) Specific binding per end-plate with ^{125}I -labelled α -bungarotoxin at 29 nm ($\bullet$), 24 nm ($\circ$), 9 nm ($\blacksquare$), 10 nm ($\square$), 5.5 nm ($\blacktriangle$), 2.8 nm ($\triangle$) and 1.4 nm (∇). Each point represents a single determination.

At that time binding to the non-end-plate region was less than 10% of the total binding at the end-plate region. The binding of ^{125}I -labelled α -bungarotoxin to the end-plate was therefore highly specific, non-end-plate binding being less than 0.1% of specific end-plate binding per unit surface area of membrane. With ^{125}I -labelled 54 nm- α -bungarotoxin the binding saturated at 66 amol of ^{125}I -labelled α -bungarotoxin per end-plate in 45 min with no further

Fig. 2. Logarithmic plots of time-course experiments (a) Semi-logarithmic plots of the fraction of unreacted sites against time with different concentrations of ^{125}I -labelled α -bungarotoxin. Symbols are the same as for Fig. 1(c), except ^{125}I -labelled 200 nM- ($\blacklozenge$) and 54 nM- ($\diamond$) α -bungarotoxin. $r = -0.96$ to -1.00 for these lines. (b) Double-logarithmic plot of first-order rate constant (s^{-1}), derived from the values for the slopes of lines in (a) plus one other experiment (result not shown), against ^{125}I -labelled α -bungarotoxin concentration. Slope = 1.03, $r = 0.99$ and $k_{+1} = (2.27 \pm 0.49) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ S.D., $n = 10$).

Fig. 3. Concentration-dependence of ^{125}I -labelled α -bungarotoxin association

(a) and (b) show the amount of ^{125}I -labelled α -bungarotoxin bound specifically per end-plate at different concentrations of ligand with 10 (a) and 135 (b) min incubations. (c) Double-logarithmic plot of first-order rate constants derived from results in Figs. 3(a) and 3(b) with 10 ($\bullet$) and 135 ($\circ$) min incubations against ^{125}I -labelled α -bungarotoxin concentration. For the 10 min incubation, slope = 0.95, $k_{+1} = 2.10 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $r = 0.99$. For the 135 min incubation, slope = 0.90, $k_{+1} = 1.74 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $r = 0.99$. The first-order rate constant was proportional to the toxin concentration over a 1000-fold range and a 10-fold time interval.

increase in specific binding at 4 h (Fig. 1*b*). The results of other time-course experiments with different toxin concentrations are shown in Fig. 1*c*). Semi-logarithmic plots of these data were linear (Fig. 2*a*) as was a double-logarithmic plot of the first-order rate constant against the toxin concentration (Fig. 2*b*), which had a unit slope (1.03). The association reaction therefore conformed to a simple bimolecular mechanism with a bimolecular-association rate constant (k_{+1}) for toxin binding of $(2.27 \pm 0.49) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ S.D., $n = 10$).

Concentration dependence of ^{125}I -labelled α -bungarotoxin association

These experiments (Figs. 3*a* and 3*b*) also gave similar saturation values of 67.3 and 57.3 amol of ^{125}I -labelled α -bungarotoxin per end-plate with

Fig. 4. Time course of ^{125}I -labelled α -bungarotoxin dissociation

The graph represents the combined normalized data from four separate experiments in which fibres were incubated in ^{125}I -labelled α -bungarotoxin (13–55 nM) for 15–45 min, washed quickly in solution A and an excess of unlabelled toxin (2–160-fold) added. Individual fibres were then removed at specified intervals for up to 17 h. k_{-1} was not greater than approx. $3 \times 10^{-6} \text{ s}^{-1}$ and $r = -0.20$.

short (10 min) and long (135 min) incubation times respectively. Double-logarithmic plots of these data (Fig. 3*c*) were linear with slopes close to unity (0.95 and 0.90) and gave essentially the same bimolecular-association rate constants, k_{+1} , of 2.1×10^4 and $1.74 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for 10 and 135 min incubations respectively. Thus the rate of toxin binding was proportional to the toxin concentration over a 1000-fold range and a 10-fold time interval, confirming a bimolecular mechanism.

Dissociation of ^{125}I -labelled α -bungarotoxin

This was followed for up to 17 h and little, if any, of the toxin was dissociated. The semi-logarithmic plot (Fig. 4) was approximately linear again suggesting a single class of sites, with a decay constant (k_{-1}) not greater than approx. $3 \times 10^{-6} \text{ s}^{-1}$ (half-time ≥ 60 h). Dissociation was unaltered by varying the initial degree of saturation at the end-plate (50–100%) or the excess of unlabelled toxin added (2–160-fold). Toxin association was therefore practically irreversible.

Slow dissociation of ^{125}I -labelled α -bungarotoxin prevented an accurate determination of the decay constant as intact fibres began to visibly degenerate after about 24 h and the dissociation seen, if any, may have resulted from proteolysis. However, there was no evidence of any rapidly dissociating component, which was also indicated by the fact that similar saturation values were found over a wide range of toxin concentrations in the association reaction. Experimentally, it would have been difficult to reliably detect or assess the significance of a small change in the slope of the semi-logarithmic plots at the end points of the time-course experiments. However, the k_{+1} derived from the independent concentration-dependence experiments with short and long incubation times were similar. This further excluded

Table 1. Summary of ^{125}I -labelled α -bungarotoxin-binding data

Experiment with ^{125}I -labelled α -bungarotoxin	Constant	Number of toxin-binding sites
Association reaction	Bimolecular-association rate constant, $k_{+1} (\text{M}^{-1} \cdot \text{s}^{-1})$	Slope of double-logarithmic plot
Time experiment	2.27×10^4	1.03
Concentration experiment (10 min)	2.10×10^4	0.95
Concentration experiment (135 min)	1.74×10^4	0.90
Competition by unlabelled toxin*		1.04
Dissociation reaction	Dissociation rate constant, $k_{-1} (\text{s}^{-1})$ Approx. 3×10^{-6}	1
Combined results of association and dissociation reaction	Equilibrium dissociation constant, K_d (i.e. k_{-1}/k_{+1}) (pM) Approx. 100	1

* See Fig. 8.

the presence of a second component and time- or occupancy-dependent changes in receptor affinity.

The results of the association and dissociation experiments (Table 1) indicated that in this experimental model there was a single class of high-affinity toxin-binding sites at the intact synapse with an equilibrium dissociation constant, K_d , of less than or equal to 100 pM.

Comparison of fibre types

In the rat the soleus muscle is slow twitch with predominantly histochemical type 1 fibres and the gastrocnemius muscle is fast twitch with predominantly histochemical type 2 fibres (see Dubowitz & Brooke, 1973). It can be seen from Figs. 5(a) and 5(b) and 7(a) and 7(b) that the specificity and affinity of toxin and tubocurarine binding did not differ between

Fig. 5. ^{125}I -labelled α -bungarotoxin binding to slow- and fast-muscle fibres

(a) Time course of ^{125}I -labelled α -bungarotoxin binding (amol bound specifically/end-plate) to slow soleus (type 1; ●) and fast gastrocnemius (type 2; ○) muscle fibres incubated in ^{125}I -labelled 38 nM- α -bungarotoxin; $k_{+1} = 1.11 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.28 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ for type 1 and type 2 fibres respectively. (b) Semi-logarithmic plot of dissociation time course of bound ^{125}I -labelled α -bungarotoxin (normalized fraction) from slow soleus (type 1; ●) and fast gastrocnemius (type 2; ○) muscle fibres. Fibres were incubated in ^{125}I -labelled 97 nM- α -bungarotoxin for 15 min, washed, and a 40-fold excess of unlabelled toxin was added. There was no ^{125}I -labelled α -bungarotoxin dissociation in this experiment.

Fig. 6. Fibre typing and radioautography

(a) Myosin ATPase reaction with acid pre-incubation (pH 4.35) showing a type 1 (dark staining) and type 2 (light staining) fibre. (b) Radioautographs of fibres where the end-plates have been lightly stained for acetylcholinesterase. The grain density is high over the end-plates and low over the non-end-plate membrane, but non-end-plate grain density increased with time (cf. 5 min and 135 min). (c) Time-course experiment with ^{125}I -labelled 5 nM- α -bungarotoxin showing grain counts per 150 μm^2 of membrane at the end-plate (e-p) and non-end-plate (Non e-p) membrane. A total of 28 fibres (16 type 1 and 12 type 2) were counted and there was no difference in grain densities between histochemical type 1 and type 2 fibres. The grains were counted under oil-immersion light microscopy at 1000-fold magnification.

these fibre types. As the soleus muscle contained some type 2 fibres radioautographs were prepared from time-course experiments on individual fibres of known histochemical type (1 and 2) and again no differences were found (Fig. 6). As with quantitative binding studies, non-end-plate grain counts increased linearly with time.

Binding of cholinergic ligands and unlabelled toxin

The properties of the acetylcholine receptors were further studied by the effect at equilibrium of cholin-

ergic ligands on the rate of ^{125}I -labelled α -bungarotoxin binding. The affinity and specificity of binding of two non-depolarizing antagonists (tubocurarine and hexamethonium) and two agonists (acetylcholine and carbachol) and a depolarizing antagonist (decamethonium) were determined. Short incubations (10–20 min) were used to ensure low amounts of non-specific binding of toxin. When toxin binding near saturation was inhibited by 90% or more, shorter fibre segments than usual (i.e. 50–100 μm) were counted for gamma radioactivity, which kept the '95/100' gamma-counting error of estimation at 5% or less.

In one series of experiments fibres were pre-equilibrated in a range of ligand concentrations at least 5 times above and below the K_i . Fibres were then incubated in the same range of ligand concentra-

Fig. 7. Inhibition of ^{125}I -labelled α -bungarotoxin binding by tubocurarine

(a) Inhibition of ^{125}I -labelled α -bungarotoxin bound specifically per end-plate (100% = amount bound in absence of tubocurarine) by tubocurarine. Fibres from the slow soleus ($\bullet$) and fast gastrocnemius ($\circ$) muscles were pre-equilibrated in tubocurarine (0.02–200 μM) for 10 min and then incubated in the same tubocurarine concentrations plus ^{125}I -labelled 100 nM α -bungarotoxin for 10 min. Symbol: $\odot$, type 1 and type 2 fibres. (b) Double-logarithmic plot of data from (a) according to $r-1 = ([I]/K_i)^h$ (eqn. 2a) where r is the ratio of ^{125}I -labelled α -bungarotoxin bound in the absence of tubocurarine to that bound in its presence. For type 1 fibres ($\bullet$) $h = 0.39$ and $K_i = 0.6 \mu\text{M}$. For type 2 fibres ($\circ$) $h = 0.43$ and $K_i = 0.8 \mu\text{M}$. $r = 0.99$ for regression line of all values. Symbol: $\odot$, type 1 and type 2 fibres.

tions plus a fixed concentration of ^{125}I -labelled α -bungarotoxin for a specified time (i.e. fixed toxin and various ligand concentrations). As the incubation times were short, the ratio r (eqn. 2a) was directly calculated from the ratio of the ^{125}I -labelled α -bungarotoxin bound in the absence and presence of the ligand, assuming this to be proportional to the ratio of the respective rate constants of toxin binding. This assumption was found to be valid by measuring the toxin uptake at saturation in some experiments and determining r from the fully integrated rate constants.

Fig. 7(a) shows the data with tubocurarine. Toxin binding was progressively inhibited as the tubocurarine concentration increased and over 90% was inhibited with 200 μM -tubocurarine. At the 100 μM - and 200 μM -tubocurarine experimental points, the same incubating conditions resulted in over 90% saturation of the receptors by ^{125}I -labelled α -bungarotoxin in the absence of tubocurarine. There was no difference between type 1 (soleus muscle) or type 2 (gastrocnemius muscle) fibres (Figs. 7a and 7b). A double-logarithmic plot of these data (Fig. 7b) according to eqn. 2(a) gave an apparent K_i of 0.6 μM and a Hill slope, h , of 0.39 for type 1

Fig. 8. Inhibition of ^{125}I -labelled α -bungarotoxin binding by cholinergic ligands and unlabelled toxin

r is the ratio of the amount of ^{125}I -labelled α -bungarotoxin bound in the absence of ligand to that bound in its presence. Fibres were pre-equilibrated for 20 min or more in a range of cholinergic ligand concentrations and then incubated in the same range of cholinergic ligand concentrations plus a fixed concentration of ^{125}I -labelled α -bungarotoxin for a fixed time. Incubation times and ^{125}I -labelled α -bungarotoxin concentrations varied between the experiments from 10 to 20 min and 49 to 60 nM respectively. Symbols: $\circ$, unlabelled toxin (no pre-incubation); $\bullet$, tubocurarine; $\blacklozenge$, acetylcholine; $\odot$, decamethonium; $\square$, carbachol; and $\blacktriangle$, hexamethonium. The correlation coefficients (r) for these lines varied from 0.89 to 1. The Hill coefficients and the apparent K_i for individual experiments are summarized in Table 2. The experiments with acetylcholine were performed in the presence of 5 μM - or 30 μM -eserine, which in control incubations did not affect toxin binding.

fibres and an apparent K_i of $0.8 \mu\text{M}$ and a Hill slope, h , of 0.43 for type 2 fibres. The low Hill coefficient, close to 0.5, was unexpected, but repeated experiments, both with and without tubocurarine pre-equilibration or calculating the ratio r from the fully integrated rate constants gave similar results.

Similar experiments on other cholinergic ligands, including hexamethonium, decamethonium, carbachol and acetylcholine (Fig. 8 and Table 2) also gave a Hill slope, h , close to 0.5. As with tubocurarine an excess of either carbachol (2mM) or the natural transmitter acetylcholine ($500 \mu\text{M}$) inhibited over 90% of the rate of radioactively labelled toxin binding near saturation.

The effect of different concentrations of unlabelled toxin on the binding of a fixed concentration of ^{125}I -labelled α -bungarotoxin is also shown in Fig. 8. Fibres were incubated in a mixture of radioactively labelled and unlabelled toxins for the same time. The ratio r was calculated and plotted as in experiments with cholinergic ligands. This experiment was close to saturation with respect to radioactively labelled and unlabelled toxin, and the slope of the double-logarithmic plot is comparable in some respects with that for cholinergic ligands (cf. eqns. 2a and 3). The unlabelled toxin completely inhibited the binding of radioactively labelled toxin and the slope (1.04) was close to 1, indicating that radioactively labelled and unlabelled toxins bound competitively at the same site by a bimolecular mechanism. In this experiment 50% inhibition (i.e. when $r-1=1$) of 49nM radioactively labelled toxin binding occurred with 30nM unlabelled toxin, implying that there was about a 2-fold difference in their bimolecular-association rate constants (k_{+1}). The finding of a slope of 1 with unlabelled toxin made it unlikely that the slope of close to 0.5

recorded for cholinergic ligands resulted from experimental error or the way the data had been analysed.

In this series of experiments (see Figs. 7a, 7b and 8 and Table 2) the Hill slope, h , was close to 0.5 for all reversible cholinergic ligands tested. This suggested that if cholinergic ligands were competitive inhibitors of toxin binding there was a heterogeneity of cholinergic-ligand-binding sites or, alternatively, that 'negative co-operative' binding of these ligands was present.

A second series of experiments was performed to distinguish between these possibilities. Fibres were pre-equilibrated for more than 20min in a fixed cholinergic ligand concentration equal to or up to four times greater than the K_i value. The fibres were then incubated in this same fixed ligand concentration plus a range of ^{125}I -labelled α -bungarotoxin concentrations (1–200nM) for a fixed time (i.e. various toxin concentrations and fixed ligand concentration). Ligand and toxin concentrations were chosen to ensure that fractional occupancies of 0.5–0.9 were achieved during ligand pre-equilibration and then subsequently by radioactively labelled toxin. The fractional occupancy for radioactively labelled toxin was determined for each experimental point and the fully integrated observed rate constant was plotted against the toxin concentration according to eqn. 2(b).

The results obtained are shown in Figs. 9(a) and 9(b) and Table 2. Inhibition of toxin binding by antagonists was consistent with a competitive mechanism as the plots remained linear although decreased in slope (Fig. 9a). With agonists the plots became non-linear (Fig. 9b), which suggested that agonist binding was possibly accompanied by conformational changes in the acetylcholine receptors, as there was an apparent increase of

Table 2. Summary of cholinergic-ligand-binding data

Experiment ... Ligand	Fixed toxin concentration with various ligand concentrations		Various toxin concentrations with fixed ligand concentration
	h	Apparent K_i (μM)	
Non-depolarizing antagonists			
Toxin	1.04*	—	—
Tubocurarine	0.39, 0.43, 0.42, 0.46	0.6, 0.8, 0.5, 0.4	0.7
Hexamethonium	0.54	387	98
Depolarizing antagonist			
Decamethonium	0.63	7	4
Agonists			
Acetylcholine	0.43, 0.38	3,3	0.9
Carbachol	0.42, 0.39	7,12	7

* Not a Hill coefficient.

Fig. 9. Inhibition of ¹²⁵I-labelled α-bungarotoxin binding by cholinergic ligands

k_{obs} is the observed rate constant. Fibres were pre-equilibrated in a fixed concentration of cholinergic antagonist (a) or agonist (b) for more than 20 min and then incubated in the same ligand concentration plus various concentrations of ¹²⁵I-labelled α-bungarotoxin (1–200 nM) for a fixed time (10 or 15 min). (a) ¹²⁵I-labelled α-bungarotoxin alone (●), and with 1 μM-tubocurarine (Δ), 10 μM-decamethonium (○) and 1 mM-hexamethonium (▲). (b) ¹²⁵I-labelled α-bungarotoxin with 5 μM- (●) and 20 μM-carbachol (○) or 3 μM- (▲) and 10 μM-acetylcholine (Δ). The experiments with acetylcholine were done in the presence of 30 μM- eserine. The apparent K_i values are summarized in Table 2.

Fig. 10. Effect of temperature on ligand binding (a) Arrhenius plot of ¹²⁵I-labelled α-bungarotoxin binding. The fibres were incubated for 10 min in ¹²⁵I-labelled 48 nM-α-bungarotoxin alone (●) and, after pre-equilibration for 15 min in tubocurarine or carbachol, in the presence of 0.5 μM-tubocurarine (◆) or 5 μM-carbachol (▲). For ¹²⁵I-labelled α-bungarotoxin alone (●) $r = -0.99$ and the slope = -4.1×10^3 . (b) shows a Van't Hoff plot. For carbachol (▲) $r = -0.98$ and the slope = -1.26×10^4 . For tubocurarine (◆) $r = -0.91$ and slope = -4.89×10^3 . The reaction enthalpy (ΔH) = $-\text{slope}/R$ where R is the universal gas constant.

agonist affinity with decreasing receptor occupancy. However, the changes in agonist affinity were not marked enough to give Hill coefficients of 0.5, which implied that for both agonists and antagonists there was a heterogeneity of binding sites.

The apparent K_i for each ligand (Table 2) was comparable in these experiments. The apparent K_i for decamethonium was more than one order of magnitude lower than the apparent K_i for hexamethonium, which was consistent with their different

specificities for ganglionic and end-plate nicotinic acetylcholine receptors. In this respect it was also shown that an excess of the muscarinic antagonist atropine (10 and 50 μM) did not affect the binding of radioactively labelled toxin to the end-plate.

Effect of temperature on ligand binding

This was studied by examining the rate of ^{125}I -labelled α -bungarotoxin binding in the presence and absence of reversible cholinergic ligands from 14°C to 37.5°C (Figs. 10a and 10b). The activation enthalpy ($\Delta H^{\ddagger} = 31.5 \text{ kJ} \cdot \text{mol}^{-1}$) and entropy ($\Delta S^{\ddagger} = -55 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) for ^{125}I -labelled α -bungarotoxin were consistent with the relatively slow rate of toxin binding compared with the rate of binding of other similar sized proteins to their putative receptors (e.g. insulin, $k_{+1} = 1.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; Cuatrecasas, 1971). Although carbachol binding was less exergonic than tubocurarine binding (for carbachol, $\Delta G^0 = -31.5 \text{ kJ} \cdot \text{mol}^{-1}$; for tubocurarine, $\Delta G^0 = -37.8 \text{ kJ} \cdot \text{mol}^{-1}$), the binding of carbachol was considerably more endothermic (for carbachol, $\Delta H^0 = +105.4 \text{ kJ} \cdot \text{mol}^{-1}$; for tubocurarine, $\Delta H^0 = +40.7 \text{ kJ} \cdot \text{mol}^{-1}$) and was accompanied by a large increase in entropy (for carbachol, $\Delta S^0 = +0.46 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, for tubocurarine, $\Delta S^0 = +0.26 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). This suggested that the agonist carbachol induced substantial conformational changes in the acetylcholine receptors. The thermodynamic difference between agonist and antagonist binding paralleled that observed in the other experiments.

The saturation value of radioactively labelled toxin binding with different rats and batches of toxin was $60.1 \pm 7.7 \text{ amol/end-plate}$ (mean $\pm$ S.D., $n = 22$) or $(3.62 \pm 0.46) \times 10^7$ receptor sites/end-plate. Assuming acetylcholine receptors are restricted to the apices of the synaptic folds (Fertuck & Salpeter, 1976) this would give a packing density of $(5 \pm 1) \times 10^4$ receptors/ μm^2 of membrane.

Discussion

The purpose of these experiments was to characterize native acetylcholine receptors intact in their functional unit, the end-plate. To do this the affinity and specificity of ^{125}I -labelled α -bungarotoxin and cholinergic ligand binding was quantitatively studied on fully exposed end-plates as toxin binding to whole muscle was slow and variable, confirming neurophysiological observations (Miledi & Potter, 1971). In comparison with studies on whole muscle, where saturation with 125–250 nm- α -neurotoxin took 1–2 h (Miledi & Potter, 1971; Barnard *et al.*, 1971; Fambrough & Hartzell, 1972; Berg *et al.*, 1972; Porter *et al.*, 1973; Chiu *et al.*, 1974; Libelius *et al.*, 1975), very similar, but less variable, saturation values were obtained after 10 min incubation of exposed end-plates in ^{125}I -labelled 200 nm- α -bung-

arotoxin. In further contrast with whole muscle, where only about 50% of toxin binding at the synapse was sensitive to excesses of tubocurarine, over 90% of toxin binding to exposed end-plates was inhibited by tubocurarine, carbachol and the natural transmitter acetylcholine.

Other closely comparable kinetic studies on rat muscle have utilized homogenized and solubilized acetylcholine-receptor extracts from normal and denervated rat diaphragm to overcome diffusion barriers (Brookes & Hall, 1975a,b; Colquhoun & Rang, 1976). Brookes & Hall (1975a) identified kinetically distinct major (70% of total, $k_{+1} = 4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 1.9 \times 10^{-6} \text{ s}^{-1}$) and minor ($k_{+1} = 1.1 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 4.8 \times 10^{-5} \text{ s}^{-1}$) components in the binding of monoiodinated ^{125}I -labelled α -bungarotoxin. The major component had similar rate constants to the single toxin-binding component observed in the present study at the intact end-plate. In agreement with the present study, Colquhoun & Rang (1976) found bimolecular binding of diiodinated ^{125}I -labelled α -bungarotoxin with homogenates ($k_{+1} = 3 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{-1} = 3 \times 10^{-6} \text{ s}^{-1}$) and solubilized acetylcholine receptors ($k_{+1} = 8 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$); but in contrast with the exposed end-plates used in the present paper and preparations by Brookes & Hall (1975b), where essentially all toxin binding was tubocurarine-sensitive, Colquhoun & Rang (1976) found that only 40% (synaptic acetylcholine receptors) and 80% (extrasynaptic acetylcholine receptors) of toxin binding was inhibited by tubocurarine. Some of this 'non-specific' toxin binding may have corresponded to non-end-plate binding, which could be directly measured on the intact fibres used in the present study. The equilibrium dissociation constant for toxin binding (i.e. $K_d = k_{-1}/k_{+1} \sim 100 \text{ pM}$) in these kinetic studies closely agreed, and also contrasted with values of 1–10 nm derived by equilibrium binding of diiodinated ^{125}I -labelled α -bungarotoxin to Triton-solubilized acetylcholine receptors from rat leg muscle (Almon *et al.*, 1974; Almon & Appel, 1976a).

An advantage of the present method was that the biological specificity of toxin binding could be evaluated pharmacologically and directly by comparing binding to the end-plate and non-end-plate membranes. This might explain some of the differences between results obtained in the present study and with rat muscle extracts. Alternatively, these differences might suggest that the membrane constraints and conformation of intact acetylcholine receptors differ from extracted acetylcholine receptors. Evidence for this has been obtained in acetylcholine-receptor extracts from rat muscle (Almon *et al.*, 1974) and the rich source of fish electric organs where homogenization (Popot *et al.*, 1976) affected the affinity and specificity of ligand binding. Furthermore, dissolution of membrane

fragments led to the appearance of interconvertible 'high' ($K_d \sim 30 \text{ nM}$), 'medium'- ($K_d \sim 100 \text{ nM}$) and 'low'- ($K_d \geq 1 \mu\text{M}$) affinity states for acetylcholine, whereas the affinity for antagonists remained reasonably constant (Changeux *et al.*, 1976). The situation in extracts is further complicated by biochemically distinct intracellular components that only bind toxin after cell disruption (Devreotes & Fambrough, 1975; Patrick *et al.*, 1977). Thus the relationship of the wide range of Hill coefficients, site numbers and binding constants for α -neurotoxins and cholinergic ligands found in extracts to receptor characteristics *in vivo* remains uncertain, although parallel neurophysiological studies correlated 'high'- and 'low'/'medium'-affinity states with 'desensitized' and native acetylcholine receptors respectively. These 'low'/'medium'-affinity states, despite species difference, also correlate best with the native acetylcholine receptors studied in the present paper.

Apparent inhibitor dissociation constants for cholinergic ligands obtained in the present study by measuring receptor occupancy agreed with neurophysiological determinations at intact end-plates (see Colquhoun, 1975), which, however, depend on the assumption that muscle responses (e.g. membrane potential or current) are proportional to receptor occupancy. They also correlated well with values obtained by toxin inhibition in acetylcholine-receptor extracts from mainly denervated rat diaphragm by Colquhoun & Rang (1976). However, they found Hill slopes close to 1, although some decrease in slope was noted with solubilized acetylcholine receptors at high cholinergic ligand concentrations. In the present study Hill coefficients close to 0.5 were found that, as further experiments indicated, resulted from a heterogeneity of cholinergic ligand sites. Alper *et al.* (1974) and Brookes & Hall (1975b) did not comment on Hill slopes in their respective studies on rat diaphragm extracts, but it would seem, from the range of cholinergic ligand concentrations required to inhibit toxin binding, that values close to 0.5 were present. Low Hill slopes for agonists were also noted by Almon & Appel (1976b) in rat leg muscle extracts. In fish electric organs, Hill coefficients varying from 2 to 0.5 have been found (see Heidmann & Changeux, 1978). The good correlation of toxin and cholinergic-ligand binding constants obtained in the present study with results in extracts made it unlikely that significant diffusion barriers, partly rate-limiting toxin binding, led to the low Hill coefficients. This contrasts with a K_i for tubocurarine of $10 \mu\text{M}$ determined by inhibition on toxin binding in whole muscle (Chiu *et al.*, 1974), where diffusion barriers are known to exist.

In contrast with antagonists, inhibition of toxin binding by fixed concentrations of agonists was not consistent with a purely competitive mechanism as there was an apparent decrease of agonist affinity

with increasing receptor occupancy. A similar result was obtained with solubilized acetylcholine receptors from *Electrophorus electricus* by Maelicke *et al.* (1977). In the present study further evidence of a difference between agonist and antagonist binding was seen in thermodynamic experiments where the binding of carbachol was accompanied by a large increase in entropy, which approached that found for denaturation reactions of proteins (chymotrypsinogen, pH 3.0, 0.01 M-chloride, 25°C , $S_d = +0.42 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$; Brandts, 1964). Maelicke *et al.*, (1977) reported large increases in entropy for both agonists and antagonists. A plausible explanation for the differences between agonist and antagonist binding observed in the present study is that agonists induced significant conformational changes in acetylcholine receptors. This idea is supported by the results of ligand-binding studies on extracted acetylcholine receptors (Colquhoun & Rang, 1976; Weiland *et al.*, 1976; Quast *et al.*, 1978; Barrantes, 1978) and physiological experiments at intact end-plates (Katz & Thesleff, 1957). In the present preparation decamethonium competitively inhibited toxin binding, which corresponded to its principal action *in vivo* as an antagonist, although it has weak agonist activity (Parsons, 1969). Thus the extent to which agonist affinity changes with receptor occupancy might explain the difference between strong and weak agonists (i.e. agonist efficacy).

The simplest interpretation of our results suggests that intact acetylcholine receptors have a homogeneous set of toxin-binding sites that are heterogeneous in affinity for reversible cholinergic ligands. For antagonists these sites are independent and non-interacting. Agonists induce conformational changes, which possibly vary with receptor occupancy in a negative co-operative manner. This might reflect 'desensitization' or an allosteric interaction of the acetylcholine-receptor recognition site with the ionophore site (Heidmann & Changeux, 1978). Whatever the mechanism, the results obtained in the present experimental model may well be relevant to the molecular events of neuromuscular transmission.

Certain biochemical and physiological properties of the muscle fibre are controlled by neural influences (for review see Gutmann, 1976) that have not been precisely identified. An example is the disappearance of extrasynaptic acetylcholine receptors from foetal myotubules after synapse formation and conversely their reappearance if the muscle fibre is later denervated. No difference was found between synaptic acetylcholine receptors from type 1 or type 2 fibres, suggesting that neurotrophic control is not expressed or primarily mediated by differences in the synaptic acetylcholine receptors at this level.

Finally it is noteworthy that the present method allows direct examination of properties of intact acetylcholine receptors, with morphological and

physiological correlations, on milligram amounts of tissue and may have application to other neurobiological studies and the investigation of human neuromuscular disorders such as myasthenia gravis.

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