Interaction of Di-iodinated $^{125}$I-labelled $\alpha$-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 $\alpha$-bungarotoxin, acetylcholine and other cholinergic ligands at room temperature (22°C). 2. The time course and concentration dependence of $^{125}$I-labelled $\alpha$-bungarotoxin association conformed to a bimolecular mechanism. In time-course experiments with different concentrations of $^{125}$I-labelled $\alpha$-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 ± 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 $\alpha$-bungarotoxin concentration. $^{125}$I-Labelled $\alpha$-bungarotoxin dissociation was first order with a dissociation constant, $k_{-1}$, less than or equal to $3 \times 10^{-4} \text{s}^{-1}$ (half-life ≥ 60 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 ± 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 $\alpha$-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 $\alpha$-bungarotoxin binding. 4. Cholinergic ligand binding was accompanied by an increase in entropy, which was greater for the agonist carbachol ($\Delta S^0 = +0.46 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) than the antagonist tubocurarine ($\Delta S^0 = +0.26 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). 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 $\alpha$-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, 1975a,b).

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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 $\alpha$-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
from denervated and normal mammalian muscle (Brockes & 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; Brockes & 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 125I-labelled α-bungarotoxin, acetylcholine and other cholinerigic 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, Na125I (sp. radioactivity 1750Ci/mmω and gamma counter checking source (129I) 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., 25% (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 Stortford, Herts., U.K. All other reagents were of analytical grade.

Preparative Methods

Preparation of toxin for experiments. α-Bungarotoxin. This was prepared 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 500mm-NaCl/5mm-sodium phosphate buffer, pH 7.0, by further ion-exchange chromatography and stored in portions at -30°C.

125I-labelled α-bungarotoxin. This was prepared in a fume cupboard at room temperature (22°C) with a standardized reaction procedure as follows: 75μl of d-glucose (0.5mg/ml), 40μl of glucose oxidase (50μg/ml) and 40μl of lactoperoxidase (100μg/ml) were added in that order to 75μg of α-bungarotoxin in 150μl of 500mm-NaCl/100mm-sodium phosphate buffer, pH 7.0. Na125I (5mCi) was then added, followed 3min later by 34μl of NaI (75μg/ml). After 12min the reaction was completed by addition of an equal volume (390μl) of 200mm-NaI/3mm-sodium phosphate buffer, pH 7.0. To separate the unreacted iodide, the reaction mixture was immediately applied to a Sephadex G-10 column (20cm×1.5cm) pre-equilibrated in a solution of 5mg of bovine serum albumin/ml of 3mm-phosphate buffer, final pH 7.3, and eluted with the same buffer in 1ml fractions at a rate of 1ml/min. The first peak (i.e. 125I-labelled α-bungarotoxin) contained over 90% of the applied radioactivity, which suggested that the labelled toxin was di-iodinated 125I-labelled α-bungarotoxin as the molar ratio of total iodide to toxin in the reaction mixture was 2:1. Subsequently the 125I-labelled α-bungarotoxin was adsorbed on a CM-Sephadex C-25 column (0.9cm×3cm) pre-equilibrated in a solution of 5mg of bovine serum albumin/ml of 3mm-phosphate buffer, final pH 7.3, washed with 25ml of this same buffer to remove the enzyme, electrostatically bound iodide and damaged radioactive protein fragments and then eluted in 1ml fractions at a rate of 1ml/min with a solution of 5mg of bovine serum albumin/ml of 150mm-sodium chloride/3mm-sodium phosphate buffer, final pH 7.3. The 125I-labelled α-bungarotoxin protein peak from this column was pooled, gamma-counted to determine the specific radioactivity (usually approx. 400Ci/mmω) 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 ionisation reaction prevented the non-specific adsorption and loss of radioactively labelled toxin.

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

Biological toxicity and specificity. Intraperitoneal injection (0.3 $\mu$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 (Macdermott 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-Ca²⁺, 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 $\alpha$-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 x 5 ml of solution A, fixed in 2.5% glutaraldehyde in 0.1 mol 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 $\mu$m). 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 $^{125}$I-gamma checking source (simulating 0.08 $\mu$Ci 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:

\[
[R] + [T] \xrightarrow{k_{+1}} [R][T] \quad (1)
\]

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

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:

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

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:

\[
r - 1 = ([I]/K_i)^h\tag{2a}\]

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:

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

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:

\[
r - 1 = ([T^*]/[T])\tag{3}\]

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}\text{I}-\text{labelled}\ \alpha\text{-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}\text{I}-\text{labelled} 200\text{nm-}\alpha\text{-bungarotoxin saturated in 10 min} (\text{Fig. 1a}).

Fig. 1. Time course of \(^{125}\text{I}-\text{labelled}\ \alpha\text{-bungarotoxin association**

Fibres were incubated in \(^{125}\text{I}-\text{labelled} 200\text{nm-} (\alpha)\) and 54\text{nm-} (\beta) \alpha\text{-bungarotoxin. The specific binding to end-plates (○) was taken to be the difference between the total (○) and end-plate binding (□). (c) Specific binding per end-plate with \(^{125}\text{I}-\text{labelled} \alpha\text{-bungarotoxin at 29nm (○), 24nm (○), 9nm (□), 10nm (□), 5.5nm (△), 2.8nm (△) and 1.4nm (△). 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 125I-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 125I-labelled 54nM-α-bungarotoxin the binding saturated at 66amol of 125I-labelled α-bungarotoxin per end-plate in 45 min with no further

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increase in specific binding at 4 h (Fig. 1b). 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. 2a) as was a double-logarithmic plot of the first-order rate constant against the toxin concentration (Fig. 2b), 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}\text{I}-\text{labelled } \alpha\text{-bungarotoxin association}**

These experiments (Figs. 3a and 3b) also gave similar saturation values of 67.3 and 57.3 amol of \( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin} \) per end-plate with short (10 min) and long (135 min) incubation times respectively. Double-logarithmic plots of these data (Fig. 3c) were linear with slopes close to unity (0.95 and 0.90) and gave essentially the same bimolecular-association rate constants, \( k_{+1} \), of \( 2.1 \times 10^4 \) 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}\text{I}-\text{labelled } \alpha\text{-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 \( \geq 60 \text{h} \)). 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 \( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin} \) 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

**Fig. 4. Time course of \( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin} \) dissociation**

The graph represents the combined normalized data from four separate experiments in which fibres were incubated in \( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin} (13-55 \text{nm}) \) 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 \( r = -0.20 \).

**Table 1. Summary of \( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin-binding data}**

<table>
<thead>
<tr>
<th>Experiment with ( ^{125}\text{I}-\text{labelled } \alpha\text{-bungarotoxin}</th>
<th>Constant</th>
<th>Number of toxin-binding sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Association reaction</td>
<td>Bimolecular-association rate constant, ( k_{+1} ) (M(^{-1})·s(^{-1}))</td>
<td>Slope of double-logarithmic plot</td>
</tr>
<tr>
<td>Time experiment</td>
<td>2.27 \times 10^4</td>
<td>1.03</td>
</tr>
<tr>
<td>Concentration experiment (10min)</td>
<td>2.10 \times 10^4</td>
<td>0.95</td>
</tr>
<tr>
<td>Concentration experiment (135min)</td>
<td>1.74 \times 10^4</td>
<td>0.90</td>
</tr>
<tr>
<td>Competition by unlabelled toxin*</td>
<td>Dissociation rate constant, ( k_{-1} ) (s(^{-1}))</td>
<td>Approx. 3 \times 10^{-6}</td>
</tr>
<tr>
<td>Dissociation reaction</td>
<td>Approx. 3 \times 10^{-6}</td>
<td>1</td>
</tr>
<tr>
<td>Combined results of association</td>
<td>Equilibrium dissociation constant, ( K_d ) (i.e. ( k_{-1}/k_{+1} )) (pm)</td>
<td>Approx. 100</td>
</tr>
<tr>
<td>and dissociation reaction</td>
<td>Approx. 100</td>
<td>1</td>
</tr>
</tbody>
</table>

* 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, Kd, 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

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

(a) Time course of 125I-labelled α-bungarotoxin binding (amo1 bound specifically/end-plate) to slow soleus (type 1; ○) and fast gastrocnemius (type 2; •) muscle fibres incubated in 125I-labelled 38 nM-α-bungarotoxin; k = 1.11 x 10⁴ M⁻¹ s⁻¹ and 1.28 x 10⁴ M⁻¹ s⁻¹ for type 1 and type 2 fibres respectively.

(b) Semi-logarithmic plot of dissociation time course of bound 125I-labelled α-bungarotoxin (normalized fraction) from slow soleus (type 1; ○) and fast gastrocnemius (type 2; •) muscle fibres. Fibres were incubated in 125I-labelled 97 nM-α-bungarotoxin for 15 min, washed, and a 40-fold excess of unlabelled toxin was added. There was no 125I-labelled α-bungarotoxin dissociation in this experiment.

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**Fig. 6. Fibre typing and radioautography**

(a) Myosin ATPase reaction with acid pre-incubation (pH 4.35) showing 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 125I-labelled 5 nm-α-bungarotoxin showing grain counts per 150 μm² 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.

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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 chol-
ergic ligands on the rate of $^{125}$I-labelled $\alpha$-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 $\mu$m) 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 $K_t$. Fibres were then incubated in the same range of ligand concentrations plus a fixed concentration of $^{125}$I-labelled $\alpha$-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 $\alpha$-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 $\mu$M-tubocurarine. At the 100 $\mu$M- and 200 $\mu$M-tubocurarine experimental points, the same incubating conditions resulted in over 90% saturation of the receptors by $^{125}$I-labelled $\alpha$-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 $K_t$ of 0.6 $\mu$M and a Hill slope, $h$, of 0.39 for type 1

**Fig. 7. Inhibition of $^{125}$I-labelled $\alpha$-bungarotoxin binding by tubocurarine**

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

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

$r$ is the ratio of the amount of $^{125}$I-labelled $\alpha$-bungarotoxin bound in the absence of ligand and toxin 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 $\alpha$-bungarotoxin for a fixed time. Incubation times and $^{125}$I-labelled $\alpha$-bungarotoxin concentrations varied between the experiments from 10 to 20 min and 49 to 60 nM respectively. Symbols: ○, unlabelled toxin (no pre-incubation); ●, tubocurarine; ★, acetylcholine; ○, decamethonium; □, carbachol; and ▲, hexamethonium. The correlation coefficients ($r$) for these lines varied from 0.89 to 1. The Hill coefficients and the apparent $K_t$ for individual experiments are summarized in Table 2. The experiments with acetylcholine were performed in the presence of 5 $\mu$M- or 30 $\mu$M-eresine, which in control incubations did not affect toxin binding.

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fibres and an apparent $K_1$ of 0.8 $\mu$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 (2 mM) or the natural transmitter acetylcholine (500 $\mu$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 $\alpha$-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 $r = 1$) of 49 nM radioactively labelled toxin binding occurred with 30 nM unlabelled toxin, implying that there was about a 2-fold difference in their bimolecular-association rate constants ($K_{1r}$). 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 20 min in a fixed cholinergic ligand concentration equal to or up to four times greater than the $K_1$ value. The fibres were then incubated in this same fixed ligand concentration plus a range of $^{125}$I-labelled $\alpha$-bungarotoxin concentrations (1–200 nM) 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

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* Not a Hill coefficient.

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$k_{obs}$ 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 $^{125}$I-labelled α-bungarotoxin (1–200 nM) for a fixed time (10 or 15 min). (a) $^{125}$I-labelled α-bungarotoxin alone (○), and with 1 μM-tubocurarine (△), 10 μM-decamethonium (●) and 1 mM-hexamethonium (▲). (b) $^{125}$I-labelled α-bungarotoxin with 5 μM-(-) and 20 μM-carbachol (○) or 3 μM-▲ and 10 μM-acetylcholine (△). The experiments with acetylcholine were done in the presence of 30 μM-exasperine. The apparent $K_i$ values are summarized in Table 2.

Effect of temperature on ligand binding

(a) Arrhenius plot of $^{125}$I-labelled α-bungarotoxin binding. The fibres were incubated for 10 min in $^{125}$I-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 $^{125}$I-labelled α-bungarotoxin alone (●) $r = -0.99$ and the slope = $-4.1 \times 10^3$. (b) shows a Van't Hoff plot. For carbachol (▲) $r = -0.98$ and the slope = $-1.26 \times 10^4$. For tubocurarine (●) $r = -0.91$ and slope = $-4.89 \times 10^3$. The reaction enthalpy ($\Delta H$) = -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 125I-
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 (ΔH° = 31.5 kJ·mol⁻¹) and entropy (ΔS° = −55 kJ·mol⁻¹·K⁻¹) for 125I-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⁺ = 1.5 × 10⁷ M⁻¹·s⁻¹; Cuatrecasas,
1971). Although carbachol binding was less exergonic
than tubocurarine binding (for carbachol, ΔG° = −31.5 kJ·mol⁻¹; for tubocurarine, ΔG° = −37.8 kJ·mol⁻¹), the binding of carbachol was considerably
more endothermic (for carbachol, ΔH° = +105.4 kJ·mol⁻¹; for tubocurarine, ΔH° = +40.7 kJ·mol⁻¹) and
was accompanied by a large increase in entropy
(for carbachol, ΔS° = +0.46 kJ·mol⁻¹·K⁻¹; for tubocurarine, ΔS° = +0.26 kJ·mol⁻¹·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 ± 7.7 amol/end-plate (mean ± s.d., n = 22)
or (3.62 ± 0.46) × 10⁷ 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 ± 1) × 10⁴
receptors/μm² 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 125I-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 125I-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 trans-
mittor 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 (Brockes & Hall, 1975a,b; Colquhoun &
Rang, 1976). Brockes & Hall (1975a) identified
kinetically distinct major (70% of total, k⁺ =
4 × 10⁴ M⁻¹·s⁻¹; k⁻ = 1.9 × 10⁻⁶ s⁻¹) and minor
(k⁺ = 1.1 × 10⁵ M⁻¹·s⁻¹; k⁻ = 4.8 × 10⁻⁵ s⁻¹) com-
ponents in the binding of moniodinated 125I-labelled
β-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
diodinated 125I-labelled β-bungarotoxin with homo-
genates (k⁺ = 3 × 10⁴ M⁻¹·s⁻¹; k⁻ = 3 × 10⁻⁶ s⁻¹) and
solubilized acetylcholine receptors (k⁺ = 8 × 10⁴ M⁻¹·
s⁻¹); but in contrast with the exposed end-plates
used in the present paper and preparations by Brockes
& 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 tubo-
curarine. Some of this 'non-specific' toxin binding
could have corresponded to 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. K₄ =
k⁻/k⁺ = 100 pm) in these kinetic studies closely
agreed, and also contrasted with values of 1–10 μM
derived by equilibrium binding of di-iodinated
125I-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 compar-
ing binding to the end-plate and non-end-plate
membranes. This might explain some of the differ-
ences between results obtained in the present study
and with rat muscle extracts. Alternatively, these
differences might suggest that the membrane con-
straints 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' \( (K_a \approx 30 \text{nm}) \), 'medium'- \( (K_a \approx 100 \text{nm}) \) and 'low'- \( (K_a \approx 1 \mu \text{m}) \) 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 \( \alpha \)-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 Brockes & 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, \( S_\text{A} = +0.42 \text{kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1} \); 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 myotubes 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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References
