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Effects of Inhibitors on the Binding of Iodinated α-Bungarotoxin to Acetylcholine Receptors in Rat Muscle

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SUMMARY

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The effects of agonists and antagonists on the rate of binding of iodo-α-bungarotoxin to rat diaphragm have been measured. Homogenates or detergent extracts of rat diaphragm muscle (usually denervated beforehand) were used so that the time course of binding would not be affected by diffusion. Noninhibitable binding (measured in the presence of a high tubocurarine concentration) was subtracted from all binding measurements. Bungarotoxin binding was apparently a bimolecular reaction, with rate constants of 2×10^6 M^{-1} min^{-1} (homogenate) and 5×10^6 M^{-1} min^{-1} (solubilized). The rate constant for binding was reduced in the presence of agonists and antagonists, the relationship between retardation and inhibitor concentration being that expected for simple competitive antagonism. The equilibrium constants for antagonists, such as tubocurarine, derived from these results were close to the values found by null pharmacological methods. The apparent K_I values for agonists were similar to the concentrations needed to block neuromuscular transmission. The onset of retardation was slower with agonists than with antagonists, which suggests that desensitization may be responsible both for retardation of bungarotoxin binding and for neuromuscular blockade. Similar K_I values were found for both homogenates and detergent extracts of denervated muscle, and similar values were found in homogenates of normal and of denervated muscle, when tubocurarine or carbachol was used as inhibitor.

INTRODUCTION

There is good evidence that α-bungarotoxin, a polypeptide from the venom of the snake *Bungarus multicinctus*, binds specifically and very firmly to acetylcholine receptors in electric tissue and at the motor end plate (1–5). This binding is inhibited by drugs that combine reversibly with acetylcholine receptors, and so can be used to study indirectly the binding of these drugs.

Because electric tissue provides a very rich source of receptor material (500–1000 pmoles/g of wet tissue in the electric organ of *Torpedo*), most studies of the inhibition of toxin binding by drugs that act on acetylcholine receptors have been done with this tissue, and much less is known about the properties of acetylcholine receptors in skeletal muscle. For the purpose of correlating the binding of drugs with their

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pharmacological effects, however, it would be useful to know more about the properties of the receptors in skeletal muscle, because far more is known about the pharmacological properties of muscle than of electric tissue. We have therefore studied the binding of labeled α-bungarotoxin by homogenates and extracts of rat skeletal muscle, and measured the inhibitory effects of various depolarizing and blocking agents in order to obtain results comparable with those obtained for electric tissue.

It is not possible to reach equilibrium short of saturation with an almost irreversible ligand such as bungarotoxin, even in the presence of inhibitors. It is therefore essential, if misleading results are to be avoided, to measure the *rate* of bungarotoxin binding to the receptors in the presence and absence of inhibitors. This means that it is necessary to use homogenized or solubilized muscle preparations, because in whole tissues the rate of binding will be distorted by the rate of diffusion of toxin through the tissue.

Various studies (6, 7) have suggested that depolarizing drugs may inhibit α-bungarotoxin or cobra toxin binding by causing desensitization (which is assumed to be associated with a change in the conformation of the receptor; see refs. 8–10) rather than simply by occupying the nondesensitized site. We have tried to test this point by measuring the rate at which the inhibition of toxin binding develops with agonists and antagonists. The effect of acetylcholine antagonists, such as tubocurarine, at the neuromuscular junction is rapid (11, 12), and it is assumed that the binding of agonists is also rapid; desensitization by agonists, however, takes seconds or minutes to develop (8, 10, 13–15), and so the rate at which inhibition of toxin binding develops is one guide to whether or not desensitization plays a necessary part in the production of the inhibition.

A recently published study of the binding of cobra toxin to a preparation of *Torpedo* electroplax membranes (16) has shown that the inhibition of toxin binding by agonists develops slowly, and the suggestion is made that this process is related to receptor desensitization.

METHODS

Preparation of [^{125}I]α-Bungarotoxin

α-Bungarotoxin was purified from freeze-dried venom of *Bungarus multicinctus* (Sigma Chemical Company), essentially as described by Mebs *et al.* (17). Venom (100 mg) was dissolved in a few milliliters of 0.05 M ammonium acetate buffer (pH 5.8) and applied to a column (60 × 2.5 cm) of Whatman CM52 carboxymethyl cellulose. The column was eluted with a linear gradient (750 ml) from 0.05 M to 0.5 M ammonium acetate. Fractions of 5 ml were collected and scanned for ultraviolet absorbance at 280 nm. α-Bungarotoxin emerged as the largest protein peak, and constituted 30–40% of the venom. The protein content was assayed against bovine serum albumin by the method of Lowry *et al.* (18). The activity of the toxin, assayed on the rat diaphragm, was the same as that found by Chang, Chen, and Chuang (19). The fractions containing α-bungarotoxin were pooled, desalted on a column of Sephadex G-10, and freeze-dried to give about 30 mg of pure toxin.

The α-bungarotoxin was labeled essentially as described by Vogel, Sytkowski, and Nirenberg (20). Typically 2–5 mCi of Na^{125}I (Radiochemical Centre) were added to 0.75 ml of a solution containing iodine monochloride (0.67 mM), HCl (33 mM), and NaCl (170 mM). This solution was cooled on ice, and to it was added 1 ml of a solution of α-bungarotoxin containing 125 nmole (1 mg) of toxin. There was thus a 4-fold molar excess of ICl over toxin. Vogel *et al.* (20) used a 2-fold excess, and obtained a mixture of mono- and diiodo-α-bungarotoxin. With the larger excess of ICl, virtually all of the product was diiodo-α-bungarotoxin, with only a trace of monoiodo derivative. The iodination reaction was stopped after 2 min by adding 0.2 ml of a solution containing 100 mM sodium thiosulfate and 100 mM potassium iodide. The unreacted iodine was then separated by passing the mixture through a column (20 × 1 cm) of Sephadex G-10 or G-25 which had been equilibrated with 3.3 mM sodium phosphate buffer (pH 7.4). The first radioactive peak, containing the labeled toxin,

was pooled and applied to a cation-exchange column (5 × 0.8 cm) of Whatman CM52 which had been equilibrated with 3.3 mM sodium phosphate buffer. After applying the sample, 40–50 ml of the same buffer were run through the column and the slightly radioactive effluent was discarded. The toxin was eluted with a gradient (120 ml) from 3.3 mM sodium phosphate buffer to 3.3 mM buffer plus 80 mM NaCl. The flow rate was 0.8 ml/min, and the fraction size, 1.6 ml. The main peak of radioactivity and protein (monitored by absorbance at 280 nm) occurred in fractions 20–25, and a much smaller peak, in which the ratio of radioactivity to protein content was half that of the main peak, occurred 10–15 fractions later (see Fig. 1). The fractions comprising the main peak were pooled, and the protein concentration was measured by the method of Lowry *et al.* (18) with bovine serum albumin as standard. The specific activity of this material corresponded to 2 atoms of iodine per toxin molecule. The two radioactive peaks were therefore taken to be diiodo- and monoiodo-α-bungarotoxin, respectively. Unlabeled α-bungarotoxin was found to come off the column slightly later than the monoiodinated derivative. The labeled material was stored at −20°.

When the labeled toxin was diluted with buffer to concentrations in the region of 100 nM, much of it was gradually lost by adsorption to the walls of the vessel. At a concentration of 300 nM in 140 nM NaCl, 30–50% of the activity disappeared in 3 hr when the solution was kept in glass test tubes at room temperature. Plastic tubes (polypropylene, polystyrene, or polycarbonate) or siliconized glass tubes adsorbed 70–85% of the material. The adsorption was completely prevented by the addition of bovine serum albumin (0.1 mg/ml), and this was routinely added to the buffer in which the binding experiments were carried out.

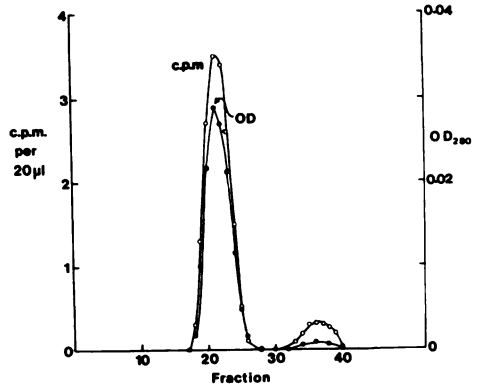

FIG. 1. *Ion-exchange chromatography of iodinated α-bungarotoxin*

Experimental details are described under METHODS. The major peak has twice the specific activity of the second peak, and they are assumed to be diiodo- and monoiodo-α-bungarotoxin, respectively. No nonradioactive toxin was present.

Preparation of Tissues

Wistar rats of 200–300 g were used. The left phrenic nerve was sectioned in the thorax under halothane anesthesia, positive pressure ventilation via a tightly fitting face mask being used during the short period when the chest was open. The rib cage was closed with a silk suture, and the skin was closed with sutures or clips. The denervated hemidiaphragms were used 7–28 days after the operation.

Homogenates were prepared by chopping denervated hemidiaphragms from 8–10 rats (1.5–2 g of tissue altogether) and homogenizing them in about 20 ml of buffered saline (120 mM NaCl plus 10 mM sodium phosphate buffer, pH 7.0) with a Polytron PT10 homogenizer at full speed for two 30-sec periods. The homogenate was kept on ice during this procedure. The homogenate was then centrifuged at 30,000 × *g* for 20 min, and the pellet was resuspended in 40 ml of buffered saline. Triton extracts were prepared by adding chopped muscle from 8–10 hemidiaphragms to 15 ml of buffered saline containing 1.5% Triton X-100. The flask was shaken at 30° for 2 hr, and the fragments of muscle were removed by straining through a gauze filter. This procedure was found to extract more than 90% of the radioactivity from muscles that had been soaked in radioactive toxin and then thoroughly washed with buffered saline before being extracted with Triton, confirming the findings of Chiu *et al.* (21).

Binding Experiments

Binding of [^{125}I]α-bungarotoxin to muscle homogenates was measured by adding to 1.0 ml of homogenate, in a 10-ml plastic centrifuge tube, 0.1 ml of the inhibitor solution (or 0.1 ml of buffered saline in the control tubes), followed 15–30 min later by 0.1 ml of [^{125}I]α-bungarotoxin, to give a final toxin concentration of 12.5 nM in most experiments. The tubes were kept in a constant-temperature water bath, and the reaction was stopped by adding 7.0 ml of buffered saline containing about 1 μM nonradioactive α-bungarotoxin. The tubes were then centrifuged at 100,000 $\times$ g for 30 min. The supernatants were removed and replaced by fresh buffered saline. The tubes were allowed to stand at room temperature for 2 days with five or six changes of buffer. The pellets were then suspended in 0.5 ml of 5% sodium dodecyl sulfate solution and transferred to vials for counting. In some experiments a well-type γ counter was used, but liquid scintillation counting, with 10 ml of NE 260 scintillator (Nuclear Enterprises), was usually used, as it gave a higher efficiency and lower background.

Control experiments showed that washing the pellets for 2 days was necessary to remove unbound radioactivity from the firmly compacted tissue pellets. If the pellets were washed for only 24 hr or less, the count rate was high and variable. Because this variation largely disappeared after 48 hr, it is likely that slow and variable loss of unbound radioactivity by diffusion accounted for the decline. In some experiments, the pellets were washed by resuspension and centrifuged again, a procedure which gave essentially the same result as prolonged washing. Loss of *bound* radioactivity was slow in suspension (half-life, 60–100 hr; see RESULTS), and should be even slower in a compact pellet containing binding sites, which would slow down diffusion to some extent. It is therefore unlikely that any serious error could have arisen from loss of bound radioactivity during the 2 days for which pellets were washed.

Binding of [^{125}I]α-bungarotoxin to Triton extracts of muscle was studied similarly by mixing 1.0 ml of extract with 0.1 ml of inhibitor solution, followed by 0.1 ml of radioactive toxin. The reaction was stopped by adding at least a 100-fold excess of nonradioactive toxin. Separation of free and bound radioactivity was achieved by passing the samples through columns (2.5 $\times$ 15 cm) of Sephadex G-75, eluting with buffered saline containing 1% Triton X-100. The flow rate was 2.5 ml/min, and 80-drop fractions were collected. The bound toxin emerged as a peak in the void volume, and was completely separated from the later peak of free toxin. The samples were counted in a liquid scintillation counter, each sample being added to 10 or 15 ml of NE 260 scintillator. The counting efficiency was measured by internal standards. Although laborious, the use of gel filtration to separate the bound toxin from free toxin was found, under the conditions of our experiments, to be much more reliable than the ion-exchange filter disc assay (22, 23). In most cases the chromatographic separation was done between 1 and 4 hr after the binding reactions, the samples being stored at 4° in the interim. Occasionally they were stored overnight. In no case was there any measurable loss of binding after storage at 4° (see RESULTS).

In all experiments, whether on homogenates or on detergent extracts, binding was expressed as femtomoles per milligram (wet weight) of muscle from which the preparation was derived.

RESULTS

Muscle Homogenates

The binding of I-BuTX[2] to a homogenate of denervated rat diaphragm is shown in Fig. 2. In this experiment the concentration of I-BuTX was 12.5 nM, and in the absence of any inhibitor the binding reached about 40 fmoles/mg, wet weight, after 3 hr. The rate of binding had flattened off greatly by this time, but no true plateau was reached even after longer incubation times. The binding was inhibited by low concentrations of tubocurarine, but was not completely prevented even by

[2] The abbreviations used are: BuTX, α-bungarotoxin; I-BuTX, iodo-α-bungarotoxin.

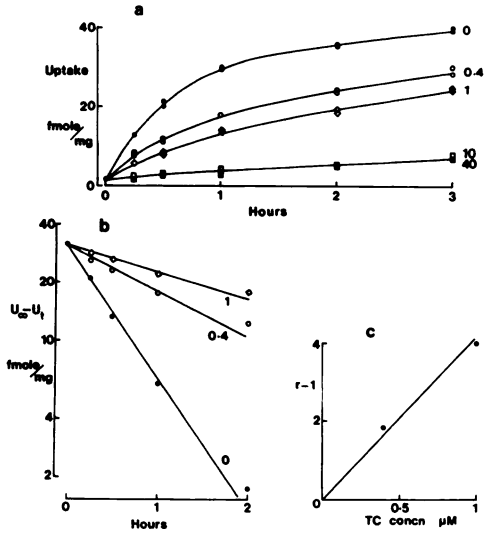

FIG. 2. *Effect of tubocurarine on binding of [^{125}I]α-bungarotoxin to denervated rat diaphragm homogenates*

a. Curves of binding *vs.* time (20°). The concentration of I-BuTX was 12.5 nM. The tubocurarine concentrations (micromolar) are shown on the right. Note that 40 μM tubocurarine produced no greater inhibition than 10 μM. This nonspecific binding was subtracted in order to measure the kinetics of specific binding.

b. Semilogarithmic plots of binding against time. U_∞ = specific binding at equilibrium; U_t = specific binding at time t. The rate constant *(k)* for toxin binding was calculated from the slope of the lines.

c. Inhibition as a function of tubocurarine (TC) concentration. The ratio r is k in the absence of inhibitor, divided by k in the presence of inhibitor. Simple competitive inhibition requires that $r - 1$ be directly proportional to inhibitor concentration.

much higher concentrations (Fig. 2). When this tubocurarine-resistant binding, measured in the presence of 40 μM or 100 μM tubocurarine, was subtracted, the residual tubocurarine-sensitive binding followed an exponential time course (Fig. 2b). The curves obtained in the presence of low concentrations (0.4 μM and 1.0 μM) of tubocurarine could be fitted by exponential curves asymptoting to the same saturation level as the control, but at a lower rate, as would be expected for a reversible inhibitor competing with an irreversible ligand (see below).

The rate constant for I-BuTX binding in the absence of any inhibitor varied with the concentration of I-BuTX. Figure 3 shows that this relationship is linear, and the slope of this line (1.8×10^6 M^{-1} min^{-1}) provides an estimate of the association rate constant k_B for the toxin-receptor reaction.

In five separate experiments in which a curve of binding against time, corrected for tubocurarine-insensitive binding, was obtained at one or more concentrations of I-BuTX, the mean value of k_B at 21° was $2.0 (\pm 0.2) \times 10^6$ M^{-1} min^{-1}.

Retardation of I-BuTX binding by agonists and antagonists. As already mentioned, high concentrations of tubocurarine do not prevent binding completely, and the tubocurarine-resistant binding (measured in the presence of 50 μM tubocurarine) amounted to up to 20% of the total binding after 3 hr of incubation in the presence of 12.5 nM I-BuTX. The level of tubocurarine-resistant binding reached

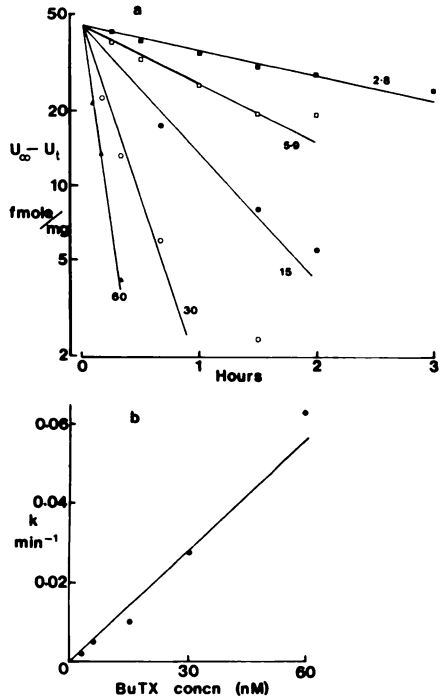

FIG. 3. *Toxin binding to denervated rat diaphragm homogenates at different concentrations of [^{125}I]BuTX*

a. Semilogarithmic plots of $U_\infty - U_t$ *vs.* time (see Fig. 2). The concentrations of [^{125}I]BuTX (nanomolar) are indicated on the curves.

b. Linear relationship between k and concentration of [^{125}I]BuTX.

(up to about 7–10 fmoles/mg) was considerably greater than the total binding measured in similar experiments on homogenates of normal muscle. It therefore represents a component that appears, or is increased, after denervation, but we have not investigated its nature further. In studying the effect of inhibitors, it was important to allow for the inhibitor-resistant component of binding, and we therefore tested whether all the inhibitors studied would depress the binding to the same maximal extent. Figure 4 illustrates an experiment in which high concentrations of tubocurarine and carbachol were tested. The concentration of each inhibitor was approximately 200 times its estimated dissociation constant (see below), which should have been enough to reduce the inhibitor-sensitive binding to a negligible level. As can be seen, the amount of binding remaining was very similar for both drugs. There was, in this and similar experiments, slightly less residual binding at high concentrations of agonists (decamethonium, suxamethonium, carbachol) than at high concentrations of antagonists (tubocurarine, hexamethonium). When the residual binding was used to calculate the degree of inhibition of the inhibitor-sensitive component, the difference was too small to matter, and we have not studied it further. In most experiments residual binding was measured in the presence of 50 μM tubocurarine, and was subtracted in order to calculate the amount of inhibitor-sensitive binding.

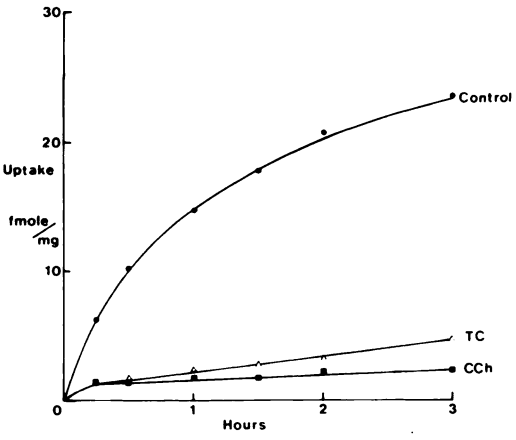

FIG. 4. *Effect of high concentrations of tubocurarine (TC, 60 μM) and carbachol (CCh, 800 μM) on binding of [^{125}I]BuTX to homogenates*

For each inhibitor the concentration was 220 × K_I, but it was consistently found that carbachol gave slightly more inhibition than tubocurarine. In most experiments binding in the presence of 50 μM tubocurarine was taken as representing "nonspecific" binding.

The simplest theory that can be envisaged to account for the retardation of binding is to postulate that (a) the inhibitory drug and I-BuTX bind to the receptor by simple bimolecular reactions, and are mutually exclusive, (b) I-BuTX binds irreversibly, (c) the inhibitor equilibrates instantaneously with the receptors not occupied by I-BuTX, and (d) diffusion of the drugs is not rate-limiting. In this case we should have

$$\frac{dp_B(t)}{dt} = k_B[B]\,[1 - p_B(t) - p_I(t)] \quad (1)$$

where $p_B(t)$ and $p_I(t)$ are the fractions of receptors occupied at time t by I-BuTX and inhibitor, respectively, k_B is the association rate constant for I-BuTX, and $[B]$ is the concentration of I-BuTX. According to assumption (c), $p_I(t) = [1 - p_B(t)]x_I/(x_I + K_I)$, where x_I and K_I are the concentration and dissociation equilibrium constant of the inhibitor. The solution of Eq. 1 using this relationship is $p_B(t) = 1 - \exp(-kt)$, where the rate constant k for exponential binding of I-BuTX is $k = k_B[B]/(1 + x_I/K_I)$. Therefore, if we define r as the ratio of the rate constant for binding of I-BuTX in the absence of inhibitor to that in the presence of inhibitor,

$$r - 1 = \frac{x_I}{K_I} \quad (2)$$

This result is formally similar to the equation for competitive drug antagonism (24, 25). A plot of $r - 1$ against x_I is predicted to be straight, with a slope equal to K_I. Figure 2c shows the results of the tubocurarine experiment plotted in this way; the equilibrium constant for tubocurarine in this experiment was 0.23 μM. Several similar experiments were done with tubocurarine and other inhibitors, and in each case the results agreed well with the theory. Dissociation constants for various

drugs estimated in this way are included in Table 1.

The main disadvantage of these experiments was that only a few inhibitor concentrations could be studied in each experiment. According to the theory, the initial rate of toxin binding should be proportional to the rate constant k. We measured the binding at 7 min, which was taken to be proportional to the initial rate, over a range of inhibitor concentrations in order to measure the dissociation constants for various inhibitors. An experiment with tubocurarine is shown in Fig. 5. As in the other experiments, tubocurarine-resistant binding amounting to about 2 fmoles/mg is evident at concentrations greater than about 20 μM, and this was subtracted from the other values. The dissociation constant obtained in this experiment was 0.29 μM. As the two methods gave very similar results, the results of initial rate experiments are included in Table 1. Logarithmic plots of $r - 1$ against inhibitor concentration for several inhibitors are shown in Fig. 6. The inhibitor dissociation constant is, from Eq. 2, the concentration causing 50% retardation of toxin binding (i.e., $r - 1 = 1$).

A few experiments were carried out with normal as opposed to denervated muscle. The amount of toxin bound was much less,

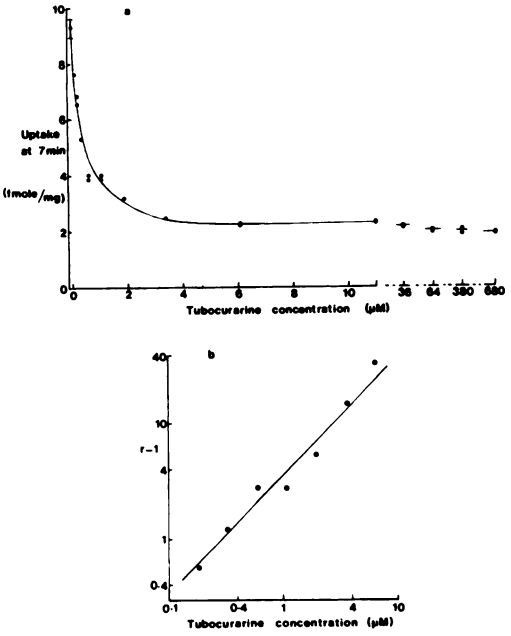

FIG. 5. *Effect of tubocurarine on initial rate of [^{125}I]BuTX binding to homogenates*

a. Uptake at 7 min in the presence of 12.5 nM [^{125}I]BuTX (closely approximating the initial rate of binding) as a function of tubocurarine concentration. Note the residual tubocurarine-resistant binding.

b. Inhibition as a function of tubocurarine concentration (logarithmic axes). r is the ratio of uptake in the absence of inhibitor to uptake in the presence of inhibitor.

TABLE 1

Equilibrium constants (K_I) for various ligands determined by retardation of I-BuTX binding, and ED_{50} values determined in rat diaphragm

Most of the values for homogenates were obtained from initial rate measurements; some experiments in which the rate constant for I-BuTX binding was determined from the whole time course of binding gave very similar results and are included in the means. The method used for measuring toxin binding rate in soluble preparations is described in the text. All experiments were done at 21–25° except those on soluble extracts, which were done at 12–14°. Experiments on acetylcholine and suxamethonium were done in the presence of 1.0 μM neostigmine. The mean and standard error are given (or the actual observations where appropriate), with the number of experiments in parentheses.

Drug	Homogenized muscle		Soluble extract (denervated), K_I	ED_{50}, normal
	Denervated, K_I	Normal, K_I		
	μM	μM	μM	μM
Antagonists				
Tubocurarine	0.22 ± 0.02 (7)	0.24, 0.25 (2)	0.26, 0.50 (2)	0.24 (2)
Hexamethonium	118 ± 20 (5)		89 (1)	640 (3)
Pancuronium	0.10, 0.11 (2)			
Agonists				
Carbachol	3.5 ± 0.6 (9)	2.3 ± 0.2 (3)	6.3 ± 1.1 (4)	6.7 (2)
Decamethonium	2.1 ± 0.2 (5)		8.6 ± 1.3 (3)	12.4 (2)
Suxamethonium	1.33 ± 0.03 (4)		2.0 (1)	2.6 (3)
Acetylcholine	0.47 ± 0.18 (4)		0.48, 0.36 (2)	

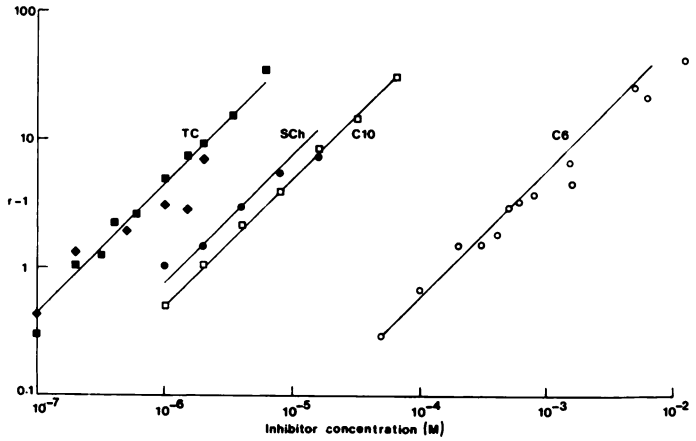

FIG. 6. *Inhibition of [^{125}I]BuTX binding to diaphragm homogenates by a series of antagonists*

The ordinate is defined as in Figs. 2c and 5b. This plot includes points obtained by measurements of rate constants as in Fig. 2, and of initial rates as in Fig. 5. The inhibitors shown are: ■, tubocurarine (TC), denervated muscle; ◆, tubocurarine, normal muscle; ●, suxamethonium (SCh), denervated muscle; □, decamethonium (C10), denervated muscle; ○, hexamethonium (C6), denervated muscle. The regression lines were drawn with a theoretical slope of unity, and the equilibrium constant K_I for each inhibitor is measured from the concentration at which $r - 1 = 1$.

roughly 2 fmoles/mg after incubation with 12.5 nM I-BuTX for 3 hr, at which time the binding was still increasing. Addition of tubocurarine (10–50 μM) reduced this by only about 40%, compared with about 80% in the experiments on denervated muscle. The absolute amount of tubocurarine-resistant binding, measured after 3 hr of incubation with 12.5 nM toxin, was, however, much smaller (0.5–1 fmole/mg) in normal than in denervated muscle (approximately 8 fmoles/mg). After subtraction of tubocurarine-resistant binding, the retardation of I-BuTX binding to normal muscle by tubocurarine (see Fig. 6), and by carbachol, gave results that were superimposable on those for denervated muscle, and consequently gave equilibrium constants (see Table 1) very similar to those found in denervated muscle.

Dissociation constants estimated for normal and denervated muscle for a number of drugs are given in Table 1.

Neuromuscular Blocking Activity

Each drug was tested on at least two different normal rat diaphragm-phrenic nerve preparations. The preparation was placed in Krebs solution bubbled with 95% O_2–5% CO_2 at room temperature (about 21°). The phrenic nerve was stimulated with supramaximal 0.1-msec stimuli at a frequency of 0.2 Hz, and the contractions were recorded isometrically. Various concentrations of drug were added to the bath and left until a steady level of blockade was achieved. The concentration (ED_{50}) needed to produce 50% inhibition of the twitch tension was interpolated from the response–log concentration curves, and is shown in Table 1.

Experiments on Triton Extracts of Denervated Muscle

Rate of I-BuTX binding. The amount of toxin bound to the solubilized receptors was estimated by gel filtration after various periods of incubation with toxin, as described in METHODS. The results (Fig. 7) show that binding followed an approximately exponential time course, as in homogenized muscle but somewhat faster. The association rate constant k_B was estimated to be 5.20 (± 0.41) × 10^6 M^{-1} min^{-1} (13 experiments) at 14°. One experiment at 25° gave k_B = 10.6 × 10^6 M^{-1} min^{-1}.

Retardation of binding by agonists and antagonists. In the presence of tubocurarine (see Fig. 7) and carbachol, the time course of binding remained approximately

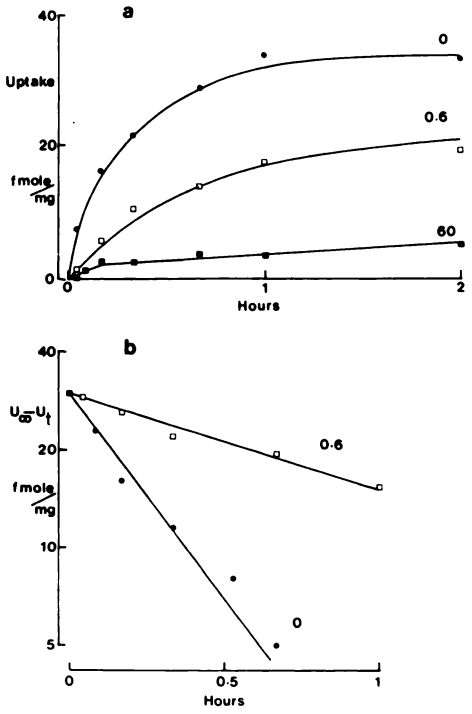

FIG. 7. *Binding of [^{125}I]BuTX to Triton extract of denervated muscle*

a. Binding as a function of time; effect of tubocurarine (micromolar concentrations shown at right). The uptake in the presence of 60 μM tubocurarine was taken to represent nonspecific binding.

b. Semilogarithmic plots, as in Fig. 2b.

exponential, with a reduced rate constant, as found in homogenized muscle. The equilibrium constants for inhibitors (K_I) could therefore be estimated as described above.

This method was fairly tedious. It was not possible to use initial rate measurements, as in the work on homogenates, because the count rates in the samples collected from the Sephadex columns were too low for accuracy. A method based on measurements of binding after 1 hr of incubation (when the controls had reached about 75% saturation) was therefore used. The amount of toxin bound, expressed as a concentration, was measured (a) after 1 hr, in the presence or absence of the test inhibitor (X_1), (b) after 1 hr, in the presence of 50 μM tubocurarine, as a measure of nonspecific binding (X_2), (c) after 5 hr in the absence of any inhibitor, at which time the specific binding was assumed to have reached saturation (X_3), and (d) after 5 hr in the presence of 50 μM tubocurarine (X_4).

The amount bound specifically at 1 hr (x) is then given by $X_1 - X_2$, and the total concentration of binding sites (b) is $X_3 - X_4$. In most experiments the initial toxin concentration was 4 nM, and the concentration of binding sites approximately 2 nM, so that the free toxin concentration could not be assumed to remain unchanged as binding proceeded. Under these conditions the rate constant for binding (k) is given by

$$k = \frac{1}{t(a - b)} \ln \left[\frac{b(a - x)}{a(b - x)}\right]$$

where a is the initial free toxin concentration, which was taken to be (total toxin concentration $- X_2$) in order to take account of the nonspecifically bound toxin. With this protocol a series of inhibitor concentrations could be studied in the experiment, and the reduction in the rate constant for BuTX binding used to determine the dissociation constant for the inhibitor in the usual way (Fig. 8). The equilibrium constants (K_I) so found are shown in Table 1.

The plot of $r - 1$ against inhibitor concentration was often slightly curved, tending to flatten at high inhibitor concentra-

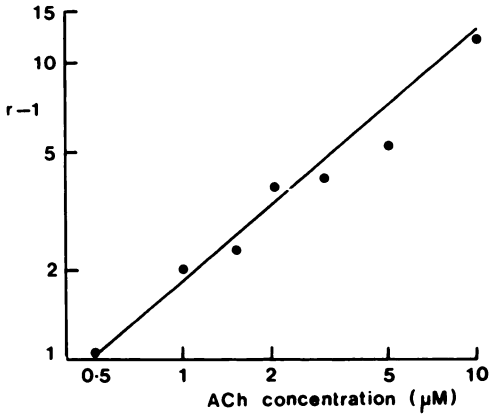

FIG. 8. *Inhibition by acetylcholine (ACh) (in the presence of 1 μM neostigmine) of [^{125}I]BuTX binding to Triton extract of denervated muscle*

The rate constants, from which the ratio r was calculated, were measured as described in the text. The slope of the line is 0.88.

tions. In 11 experiments the mean slope of the plot of log $(r - 1)$ against log (inhibitor concentration) was 0.86 ± 0.04, instead of the theoretical value of 1.0. This was not seen in the experiments on homogenates, and the reason is not clear. It could be due to a second class of binding sites with a lower affinity for the inhibitors than the specific sites. To test this, an experiment was carried out in which the elution pattern of the extracted labeled material, on a column of Sepharose 6B gel, was compared in preparations labeled in the presence and absence of 10 μM tubocurarine (Fig. 9). Had a second, less tubocurarine-sensitive site been present, this should have still been partly labeled in the presence of 10 μM tubocurarine, whereas receptor labeling would have been almost completely inhibited. The elution patterns showed no marked difference, however, and give no support to the idea that a second class of site exists. Because gel filtration tends to give broad and ill-defined peaks with Triton-solubilized membrane proteins (2, 26), the test is not a conclusive one.

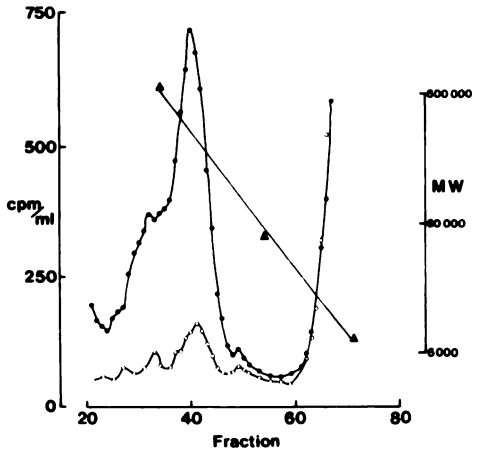

FIG. 9. *Gel filtration of Triton extract of denervated muscle labeled with [^{125}I]BuTX*

The extract was labeled with 15 nM [^{125}I]BuTX for 15 min with no inhibitor present (●), and in the presence of 10 μM tubocurarine (○). The column (2.5 × 90 cm) of Sepharose 6B was equilibrated with buffered saline containing 1% Triton X-100. The fraction volumes were 5 ml. A large peak of radioactivity at fraction 71 corresponds to free [^{125}I]BuTX, and the positions of the peaks obtained with thyroglobulin (mol wt 670,000) and ovalbumin (mol wt 48,000) were used for calibration (▲). The main labeled peak corresponds to 300,000 mol wt.

Rate of Inhibitor Action

Experiments were done to measure the rate of development of the inhibitory effect of agonists and antagonists on I-BuTX binding to homogenates of denervated muscle. These rates were fast enough to necessitate use of the shortest practicable incubation with I-BuTX, and only one measurement could be made before equilibrium was reached between inhibitor and receptor.

The initial rate of I-BuTX binding, during an incubation of 30 sec (or occasionally 60 sec) with toxin, was measured with inhibitor added (a) simultaneously with the I-BuTX (zero preliminary incubation with inhibitor), and (b) 10–15 min before the I-BuTX (effectively infinite preliminary incubation with inhibitor). The results in Fig. 10 show that tubocurarine was hardly more effective after incubation with the tissue for 10 min than when added simultaneously with the toxin, whereas carbachol was substantially more effective in the former case; i.e., the retarding effect of carbachol developed more slowly than that of tubocurarine.

If certain assumptions are made, it is possible to calculate from these experiments a rate constant for the interaction between inhibitor and receptor. Suppose that, whatever the mechanism of receptor protection may be, the fraction of free receptors (i.e., those to which toxin can still bind), $p_f(t)$, falls exponentially, with rate constant k_I, after addition of the inhibitor. In this case

$$p_f(t) = p_f(\infty) + [1-p_f(\infty)]e^{-k_I t} \qquad (3)$$

The incubation with I-BuTX is very brief in relation to the time constant for toxin binding, and so the receptor occupancy by toxin will always be very small (less than 0.04 with 40 nM toxin for 30 sec) and may be neglected. Therefore the rate of toxin binding (p_B) will be

$$\frac{dp_B(t)}{dt} = k_B[B]p_f(t) \qquad (4)$$

When inhibitor and toxin are added simul-

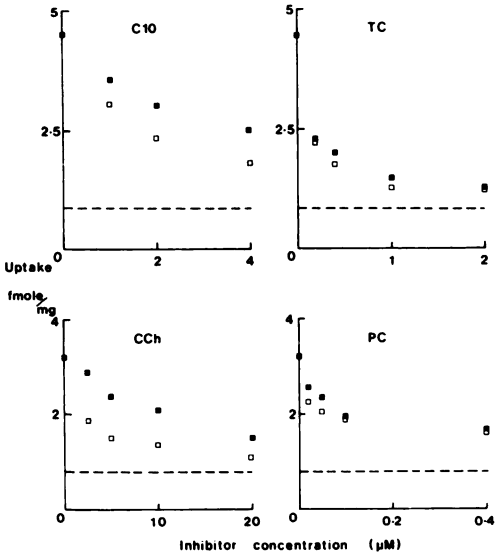

FIG. 10. *Rate of onset of inhibition of toxin binding to muscle homogenates by different inhibitors*

The total binding 30 sec after the addition of 40 nM [^{125}I]BuTX was measured when the inhibitor had been added 10–15 min previously (□) or simultaneously with the labeled toxin (■). Nonspecific binding (- - -) measured in the presence of 50 μM tubocurarine. Inhibitors tested were decamethonium (C10), carbachol (CCh), tubocurarine (TC), and pancuronium (PC). The two agonists, decamethonium and carbachol, were less effective when added simultaneously with the toxin, whereas the antagonists were almost as effective when no preliminary incubation was used.

taneously (zero preliminary incubation time), the occupancy by toxin that results, after a brief (30 or 60 sec) time t should be given by solving Eqs. 3 and 4. This yields the result in Eq. 5.

$$p_B(t) = k_B[B]\left[p_f(\infty)t + \frac{1}{k_l}(1 - p_f(\infty))(1 - e^{-k_l t})\right] \quad (5)$$

On the other hand, if the inhibitor has already equilibrated before the addition of toxin (10–15-min preliminary incubation), so that the fraction of available receptors is reduced to $p_f(\infty)$, then the occupancy (p_B^*) resulting from a brief incubation, of duration t, with the toxin will be

$$p_B^*(t) = k_B[B]p_f(\infty)t \quad (6)$$

Combining Eqs. 5 and 6 gives the ratio of occupancies by toxin, following zero and 15 min of preliminary incubation with the inhibitor, as shown in Eq. 7.

$$\frac{p_B(t)}{p_B^*(t)} = 1 + \left(\frac{1 - e^{-k_l t}}{k_l t}\right)\left(\frac{1 - p_f(\infty)}{p_f(\infty)}\right) \quad (7)$$

This equation was solved numerically for k_l, using (a) the observed ratio of the uptakes with zero and with 10–15 min of preliminary incubation with inhibitor as an estimate of $p_B(t)/p_B^*(t)$, and (b) the value of $p_f(X)$ calculated as the ratio of the amount of toxin bound after 10–15 min of incubation with inhibitor to the amount bound in the absence of inhibitor (see Eq. 6). All values for binding were corrected by subtraction of the amount of noninhibitable binding, estimated as the binding in the presence of 50 μM tubocurarine.

It was found that k_l increased with inhibitor concentration, but this relationship was not explored in detail. Most measurements were made at a single inhibitor concentration, chosen to be close to the observed equilibrium constant (K_I) value (see Table 1).

The values for k_l found for agonists at 25° are shown in Table 2. It can be seen that, for all four agonists tested, the time constant for onset of their retarding effect on toxin binding was in the range of 12–17 sec, far slower than would be expected for simple receptor occupancy.

On the other hand, the duration of preliminary incubation with antagonist inhibitors had only a very small effect on the amount of I-BuTX bound. In other words, the onset of their retarding effect on I-BuTX binding was too fast to be measured by the present method. In some cases the toxin uptake was less (although not significantly so) when the inhibitor was added simultaneously with the toxin than when it was added 15 min earlier, implying an effectively infinite rate of onset of the inhibitor effect (denoted ∞ in Table 2). The values of k_1 observed (min^{-1}), again at an inhibitor concentration close to K_I, are

TABLE 2

Rate constants (k_I) for onset of retarding effect of various ligands on initial rate of I-BuTX binding

Initial rates were measured by incubation of denervated muscle homogenates with 40 nM I-BuTX for 30 sec at 25°. Experiments on acetylcholine and suxamethonium were done in the presence of 1.0 μM neostigmine. The ratio p_B/p_B^* was estimated as the ratio of specific (i.e., after subtraction of tubocurarine-resistant uptake) uptakes measured (a) when the inhibitor was added simultaneously with the toxin and (b) when the inhibitor had been added 10 min previously (see text). The larger value for agonists than antagonists reflects the slower action of the agonists. The mean and standard error are given (or the actual observations where appropriate), with the number of experiments in parentheses.

Drug	p_B/p_B^*	k_I	
		min^{-1}	
Antagonists			
Tubocurarine (0.2 μM)	1.04 ± 0.05	18.6, 15.3, ∞	(3)
Hexamethonium (120 μM)	0.90	104, ∞	(2)
Pancuronium (0.1-0.15 μM)	1.10	17.4, 26	(2)
Gallamine (1-6 μM)	1.11	13.4, 13.5	(2)
Agonists			
Carbachol (2.5-3 μM)	1.62 ± 0.12	4.0 ± 1.1	(4)
Decamethonium (2.0 μM)	1.36 ± 0.06	3.7 ± 1.0	(4)
Suxamethonium (1-1.3 μM)	1.53 ± 0.06	3.6 ± 0.4	(3)
Acetylcholine (0.6 μM)	1.94	4.9, 5.3	(2)

shown in Table 2. The longest time constant for any of the four antagonists tested was 4.5 sec, and the experimental errors (when using a 30-sec incubation with toxin) are such that it is not possible to conclude that there is any measurable delay with the antagonists, in contrast with the relatively slowly developing inhibition found with the agonists.

Rate of Dissociation of BuTX

Our experiments necessitated leaving an interval of time between completing the incubation and measuring the amount of bound toxin, and it was necessary to check whether any dissociation of bound toxin was likely to have occurred during this period. Recent work (27, 28) has suggested that part of the bound toxin may dissociate quite rapidly.

To test for dissociation of toxin from homogenates, the homogenate (approximately 50 mg of tissue per milliliter) was labeled to about 80% saturation by incubation with 7 nM [^{125}I]BuTX for 2 hr. The homogenate was then diluted 10-fold with buffer containing 380 nM nonradioactive BuTX, to give about a 500-fold excess of nonradioactive toxin, and kept at room temperature. Aliquots (17 ml) were taken at intervals for up to 45 hr and centrifuged. The pellets were rinsed briefly and then extracted with 5% sodium dodecyl sulfate for counting.

In similar experiments on Triton-extracted receptors, an extract containing the equivalent of about 100 mg of tissue per milliliter was labeled with 4 nM [^{125}I]-BuTX for 4 hr. A 500-fold excess of nonradioactive toxin was then added, and the temperature was kept at 14°. Samples were taken at intervals and applied to a Sephadex column as described earlier.

The rate of dissociation of toxin from homogenates was slow (Fig. 11). The rate constant calculated from a semilogarithmic plot was 0.010 hr^{-1} and 0.017 hr^{-1} in two experiments. During the 48 hr of washing used for the centrifugation assay, a maximum of about 30% of the toxin might therefore have dissociated. Since dissociation from the Triton-extracted receptor preparations was negligible up to 45 hr (Fig. 11), no error would have arisen from the varying interval between stopping the reaction and the chromatographic separation process.

DISCUSSION

Kinetics of Binding and Dissociation of BuTX

In agreement with studies on the binding of neurotoxins to acetylcholine receptors in electric tissue (29-32), it was found

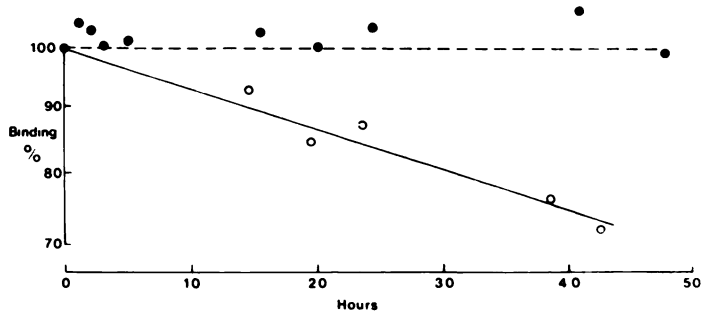

FIG. 11. *Rate of dissociation of [^{125}I]BuTX from muscle homogenates (○) and from Triton extract (●)* The binding at zero time is shown as 100% on the ordinate, which is scaled logarithmically.

that the binding approached saturation exponentially, and that the rate constant for binding was proportional to the toxin concentration, allowing an association rate constant to be calculated. The level of binding finally achieved, after correction for "nonspecific" binding (measured in the presence of 50–200 μM tubocurarine), did not vary with the toxin concentration, suggesting that the dissociation rate was so small that, at the concentrations used (4–12.5 nM in most experiments), the equilibrium level of binding approached 100%.

Measurements of the rate of dissociation agreed with this conclusion; with Triton-extracted receptors, no dissociation could be detected over 45 hr, whereas, with homogenates, the dissociation rate constant appeared to be about 2×10^{-4} min^{-1}. With the estimated association rate constant for the homogenates (2×10^{6} M^{-1} min^{-1}) this figure would imply that the equilibrium constant should be about 10^{-10} M, so that the concentrations used should have produced more than 90% saturation. Actually, because there is no evidence that the decline of bound activity with time represents reversible dissociation of toxin molecules rather than gradual proteolytic degradation of the preparation, the true rate of dissociation could be even slower than the results indicate.

The association rate constants are somewhat less than those found by other workers in similar experiments (see ref. 30), but the only closely comparable study is that of Brockes and Hall (27), who measured the kinetics of binding of monoiodo-BuTX (not diiodo-BuTX as used in the present work) to crude Triton extracts of denervated muscle, as well as to purified preparations of receptors. They identified two kinetically distinct components of binding, the slower one constituting about 75% of the total binding in the crude Triton extract. The dissociation rate constant for this component was very small (about 3×10^{-5} min^{-1}), and the association rate constant was 7.2×10^{6} M^{-1} min^{-1} at 35°, which is similar to our value (5.2×10^{6} M^{-1} min^{-1} at 14°) for extracts of muscle. Brockes and Hall (27) also obtained evidence of a faster component dissociating with a half-time of about 4 hr and amounting to 20–25% of the total binding in crude extracts of muscle. We did not find any evidence for the existence of this component in our preparations. Our results do not agree with those of Alper, Lowy, and Schmidt (33), who found that the reaction of [^{125}I]BuTX with Triton-extracted receptors from denervated muscle reached 40% saturation after 10 min at room temperature in the presence of 40 pM toxin. The association rate constant implied by these results is about 1.5×10^{9} M^{-1} min^{-1}, which is larger than our results by a factor of 300, and out of line with similar measurements in other systems (30). It is also hard to reconcile the present results, or those of Brockes and Hall (27), with the conclusion of Almon, Andrew, and Appel (34) that the equilibrium constant for I-BuTX measured on extracts of denervated rat leg muscles is 1.25–1.42 nM. They did not measure rates of reaction, but it is clear that the association would have to be very much slower, or dissociation much faster, than we and others have found to give an equilibrium constant as large as this.

Mechanism of Receptor Protection by Agonists and Antagonists

The type of inhibition of toxin binding produced by both agonists and antagonists is consistent with a simple competitive mechanism, in that the inhibitors affect the rate of equilibration with toxin without reducing the level of binding ultimately reached, and also in the linear relationship between the degree of inhibition (expressed as $r - 1$) and the inhibitor concentration. We have not found any evidence for the more complex type of inhibition (hyperbolic competitive inhibition) described by Reich and his colleagues (31) in studies of the effect of tubocurarine on the binding of cobra toxin to receptor preparations from electric tissue. With agonists, however, the inhibition of toxin binding developed much more slowly than can reasonably be accounted for by the rate of association of the agonist with the receptors (which is assumed to be a very rapid process). The apparently simple competitive interaction may therefore be misleading, and it is quite possible (although perhaps unlikely) that a more complex mechanism is also responsible for the effect of antagonists.

The dissociation constant (0.25 μM) that we obtained for tubocurarine agrees well with pharmacological estimates on various neuromuscular preparations, which fall within the range of 0.11–0.73 μM (see ref. 35), and we also found no difference between the dissociation constant for tubocurarine in normal and denervated muscle, in contrast to previously reported findings. Beranek and Vyskocil (36) found that end plate potentials and iontophoretic acetylcholine potentials elicited close to the end plate of normal rat diaphragm fibers were reduced 50% by a very low tubocurarine concentration (about 0.03 μM), whereas 50% inhibition of extrajunctional acetylcholine potentials in denervated muscle required about 0.2 μM, which is close to the value for the equilibrium constant for tubocurarine determined by pharmacological methods for both extrajunctional and junctional receptors. Diffusional factors were largely excluded as a cause of this difference, which seemed most likely to reflect a true difference in the affinity constant for tubocurarine for the two types of receptor, but subsequent estimates, in which null methods were used, have not revealed any differences (see ref. 35). Brockes and Hall (28) have obtained evidence, from purified receptor preparations, that the isoelectric point for the junctional receptors is slightly (0.15 pH unit) lower than for extrajunctional receptors, and that their affinity for tubocurarine as measured by inhibition of I-BuTX binding differs by approximately the amount expected from the findings of Beranek and Vyskocil (36). It appears, however, from Figs. 5 and 6 in ref. 28, that the difference in the action of tubocurarine on the two types of receptor was confined to tubocurarine concentrations below 0.1 μM, giving less than 30% inhibition of toxin binding. The concentration required for 50% inhibition (approximately 0.2 μM) is, however, about the same for junctional and extrajunctional receptors, and the difference between the two inhibition curves comprises only a fairly small deviation at low tubocurarine concentrations, rather than the over-all shift that would be expected if there were a true difference in affinity. A similar study by Alper *et al.* (33) showed no difference between extracts of normal and denervated muscle, both giving 50% inhibition of toxin binding at very low concentrations of tubocurarine (0.01–0.012 μM). Two recent reports (37, 38) on the actions of tubocurarine and BuTX on rat and mouse muscle have given some support to the view that junctional and extrajunctional receptors differ quantitatively in their pharmacological properties. In these experiments acetylcholine responses, end plate potentials, and BuTX binding were studied in intact muscles, and difficulties in interpretation arise because of the relatively low rates of diffusion and the varying receptor density in different regions of the fiber. This makes it difficult to be sure, for example, that differences in the ability of tubocurarine to protect against the blocking action or binding of BuTX represent actual differences in the properties of the receptors and not merely differences in their distribution (see refs. 35, 39, 40).

The results in Table 1 show that the apparent K_I values for ligands measured on soluble extracts of denervated muscle are very similar to the values found when muscle homogenates were used. The only discrepancy of any size occurred with decamethonium, which had an apparent affinity that was about 4 times lower for the solubilized receptor than that in homogenized tissue. These results suggest that the process of solubilization did not change the ligand binding site in any drastic way (cf. refs. 29, 41, and 5).

The results shown in Table 1 enable the potency of drugs as neuromuscular blocking agents to be compared with potency in inhibiting I-BuTX binding. The ED_{50} for tubocurarine is similar to its dissociation constant, which suggest that the margin of safety for neuromuscular transmission at the temperature at which these experiments were done is lower than has been found under more physiological conditions (42, 43). On the other hand, the ED_{50} for hexamethonium (640 μM) was 5.4 times larger than its K_I (118 μM); this difference is in the direction expected from the evidence that hexamethonium, but not tubocurarine, has time to dissociate and to approach a competitive equilibrium during the brief period in which the released acetylcholine remains in the synaptic cleft (44, 45).

For agonists, as well as antagonists, the ED_{50} for blocking neuromuscular transmission lies close to the K_I value. It might have been expected that a full agonist such as carbachol would depolarize the end plate sufficiently to block transmission at concentrations at which the receptor occupancy was very small, whereas a partial agonist such as decamethonium would have to occupy a substantial fraction of the receptors in order to depolarize the end plate enough to block transmission. This would lead to the prediction that the ED_{50}/K_I ratio should be much smaller for carbachol or suxamethonium than for decamethonium. Such a difference was not found, however, the ED_{50}/K_I ratio being quite close to unity for all three drugs. A possible explanation for this is that the same process—desensitization—is responsible both for the inhibition of toxin binding and for the neuromuscular blockade. Thesleff (46) showed that the depolarization produced by decamethonium in rat muscle subsided fairly quickly while the transmission blockade persisted indefinitely, and concluded that desensitization was responsible for the prolonged blockade. In this connection, our finding that the inhibition of toxin binding by agonists develops relatively slowly suggests that a process slower than receptor occupation is responsible for the inhibition. Desensitization at cholinergic synapses develops at a rate (7–9, 13) comparable to that which we observed for the inhibition of toxin binding, i.e., with a time constant of 10–15 sec, and Lester (7) has suggested that desensitization at the frog motor end plate protects the receptors against blockade by cobra toxin. A similar conclusion has been drawn from recent studies of toxin binding and desensitization in electroplax preparations (16, 47). The most commonly held view of the mechanism of desensitization is that it results from a slow conformation change of the receptors, followed by a similarly slow reversion to normal when the agonist is removed. If it is assumed that desensitization by agonists occurs because they have a higher affinity for desensitized than for normal receptors, the gradual appearance of desensitization will be accompanied by increasing agonist occupancy, and a consequent decrease in the number of receptors available for combination with toxin. Thus a slow onset of inhibition could occur even if the toxin molecules do not themselves discriminate between normal and desensitized receptors. If this is the case, the inhibition should disappear as soon as the agonist is removed, before the desensitized receptors have reverted to normal, whereas if the toxin molecules fail to bind to the desensitized receptors, recovery from inhibition should be slow. We could not, for technical reasons, measure the rate of recovery from inhibition with any certainty, and have not been able to answer this question.

Irrespective of the exact mechanism responsible for the slow development of inhibition by agonists, it is obvious that care is needed in interpreting the values for agonist dissociation constants that are ob-

tained from measurements of toxin binding. If it is assumed that the inhibition is associated with a fairly slow conformation change ($R \rightarrow R'$) in the receptors, as has often been suggested as a mechanism for desensitization (8–10), then the affinity of the agonist for R' must be greater than its affinity for the native conformation, R. Within the limits of experimental error, our results indicate no more than a single binding constant for agonists, which suggests [see discussion by Colquhoun (48)] that the measured dissociation constant will approximate the dissociation constant for the desensitized conformation, and the affinity for the native conformation, which presumably predominates under physiological conditions, will be a good deal lower. The estimates of agonist dissociation constants given in Table 1 are therefore likely to be substantial underestimates of the dissociation constants for native receptors. Direct measurements of agonist binding (41) will give a similar underestimate if a slow, agonist-induced conformation change takes place. It is possible that this accounts for the very low dissociation constant (8 nM) for acetylcholine measured on *Torpedo* receptors, which is hard to reconcile (see ref. 5, p. 355) with the rapidity of action of endogenously released acetylcholine. The kinetic inconsistency would be explained if binding studies measure the affinity of acetylcholine for desensitized receptors, while synaptic function is related to its much lower affinity for native receptors.

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