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Binding of *Naja nigricollis* [^{3}H]α-Toxin to Membrane Fragments from *Electrophorus* and *Torpedo* Electric Organs

II. Effect of Cholinergic Agonists and Antagonists on the Binding of the Tritiated α-Neurotoxin

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SUMMARY

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The effects of nicotinic effectors on the kinetics of association and dissociation and on the binding at equilibrium of a tritiated α-neurotoxin from *Naja nigricollis* with membrane fragments purified from *Electrophorus* and *Torpedo* electric organs are studied. Increasing concentrations of nicotinic agonists and antagonists decrease the initial rate of [^{3}H]α-toxin binding to membrane fragments from both species. This rate becomes negligible at high concentrations of effectors. The "protection curves" obtained are compared with the binding curves of radioactive effectors to the same membrane fragments. In the case of *Torpedo* membrane fragments, the binding curve of [^{3}H]-acetylcholine (in the presence of *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate) is slightly sigmoid (n_H = 1.3); half-saturation occurs at 8 nM acetylcholine. [^{3}H]-Decamethonium binds to one class of sites with K_D = 0.8 μM, and possibly to another class with lower affinity. The binding of both acetylcholine and decamethonium is competitively inhibited by *d*-tubocurarine (K_D = 0.2 μM) and completely displaced by α-toxin. The numbers of decamethonium and acetylcholine binding sites on *Torpedo* membrane fragments are very close to the number of [^{3}H]α-toxin binding sites. The binding curves of decamethonium and acetylcholine can be superimposed on the "protection curves" of these two agonists against [^{3}H]α-toxin binding. The data are interpreted on the basis of a mutual exclusion of cholinergic effectors and α-toxin from a common site, which is identified as the nicotinic receptor site. Comparison of our protection data with the binding data of Kasai and Changeux [(1971) *J. Membr. Biol.*, **6,** 1–80] shows that the same result holds in the case of *Electrophorus* membrane fragments. The dissociation constants of a large spectrum of cholinergic effectors were determined by following the protection against [^{3}H]α-toxin. The pharmacological properties of the receptors from *Electrophorus* and *Torpedo* appear different. In the case of *Electrophorus*, the dissociation constants for all the agonists and antagonists coincide with the "apparent dissociation con-

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stants" measured *in vivo* on the electroplax. At equilibrium decamethonium and *d*-tubocurarine displace [^{3}H]α-toxin from *Electrophorus* membrane fragments; the data are in given domains of concentrations, compatible with the hypothesis of a mutual exclusion of α-toxin and cholinergic effectors from the same binding site. However, higher concentrations of both ligands enhance the rate of dissociation of [^{3}H]α-toxin from its membrane site.

INTRODUCTION

One of the most interesting aspects of the mode of action of snake venom α-toxins is that cholinergic effectors block their interaction with their postsynaptic target. This was seen *in vivo* by Lee and Chang (1) with *d*-tubocurarine and further demonstrated *in vitro* by Changeux *et al.* (2) by following $^{22}Na^+$ permeability of excitable membrane fragments and by Miledi *et al.* (3) in their binding studies with α-bungarotoxin and *d*-tubocurarine or carbamylcholine. These early observations were subsequently extended to a wider spectrum of cholinergic ligands (4). The parallelism observed between the potency of a given effector as a cholinergic ligand on *Electrophorus* electroplax and its potency as a protecting agent against *Naja nigricollis* [^{3}H]α-toxin binding to excitable membrane fragments strengthened the view that this α-toxin binds with a high selectivity to the cholinergic receptor site.

We present here quantitative data on the effect of a variety of known cholinergic agonists and antagonists on *N. nigricollis* [^{3}H]α-toxin binding to membrane fragments from *Electrophorus* and *Torpedo*. We confirm that, over a wide concentration range, [^{3}H]α-toxin labels a population of membrane sites which bind cholinergic ligands as well. Equilibrium dissociation constants of several nicotinic ligands for this class of sites are determined. In the case of *Electrophorus*, they are found to be very close to the "apparent" dissociation constants determined with electroplax and excitable membrane fragments (5). This class of sites is therefore interpreted as the locus of electrogenic action of acetylcholine. The binding properties of these sites appear different in *Torpedo* and *Electrophorus*. Several kinetic and equilibrium properties of the interaction of [^{3}H]α-toxin with its membrane site are presented and discussed.

METHODS

Binding of [^{3}H]α-Toxin to Electrophorus and Torpedo Membrane Fragments in the Presence of Cholinergic Effector

Membrane fragments from fresh electric organs of *E. electricus* and *T. marmorata* were prepared as described in the preceding paper (6). [^{3}H]α-Toxin binding was followed by Millipore filtration. In general, the membrane fragments were incubated for 10 min at room temperature in Ringer's solution supplemented with the desired concentration of effector before the reaction was started by adding [^{3}H]α-toxin. In reversal experiments, a small volume (less than 5% of the volume of the reaction mixture) of concentrated effector solution was added to the reaction medium. It was determined that high concentrations of cholinergic effector do not significantly change the background counts retained on the filters.

Reversible Binding of Tritiated Cholinergic Effectors to Torpedo Membrane Fragments

Binding was measured by the ultracentrifugation method of Kasai and Changeux (5). The membrane suspension was diluted 2-fold (for [^{3}H]decamethonium binding) or between 3- and 25-fold (for [^{3}H]acetylcholine binding) in a medium which had the following final composition: 250 mM NaCl, 5 mM KCl, 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM phosphate buffer, pH 7.0. Aliquots (250 μl–4 ml) were distributed in polycarbonate tubes and eventually incubated at room temperature with unlabelled cholinergic effector or α-toxin for about 1hr. When the binding of acetylcholine was measured, the membrane fragments were initially incubated for 40 min in the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate (7). Under these conditions no hydrolysis of acetylthiocholine could be measured by the

Ellman method. [*methyl*-^{3}H]Decamethonium chloride (400 Ci/mole) or [*N-methyl*-^{3}H]-acetylcholine chloride (120 Ci/mole) was then added, and the preparation was kept at room temperature for an additional hour. Total and free ligand concentrations were estimated before and after centrifugation at 100,000 × *g* for 1 or 2 hr by counting aliquots in 10 ml of Bray's scintillation liquid. The yield of counting depended on the volume of the aliquot added to Bray's solution, and varied from 42% (0.1 ml) to 27% (1 ml).

Responses of Electrophorus Electroplax and Excitable Microsacs in Vivo and in Vitro

Steady-state decreases of membrane potential resulting from bath application of cholinergic agonists were followed by the method of Higman, Podleski, and Bartels (8) with the isolated electroplax dissected from the Sachs organ of large eels. The response of excitable microsacs *in vitro* was measured by the method of Kasai and Changeux (5) by following $^{22}Na^+$ efflux.

Sources of Chemicals

Decamethonium bromide, *d*-tubocurarine chloride, hexamethonium bromide, nicotine hydrochloride, carbamylcholine chloride, and atropine sulfate were obtained from K & K Laboratories; acetylcholine bromide and choline chloride, from Eastman Kodak; gallamine triethiodide, from Specia, Paris. Nicotine base was a gift from Dr. J. Jacob, Institut Pasteur, and muscarone, from Professor P. G. Waser, Zurich. [*methyl*-^{3}H]-Decamethonium chloride (400 Ci/mole) and [*N-methyl*-^{3}H]acetylcholine chloride (120 Ci/mole) were obtained from the Radiochemical Centre.

RESULTS

Figure 1 shows the effects of the cholinergic agonist decamethonium on the complete time course of the reaction of [^{3}H]-α-toxin with excitable membrane fragments from *E. electricus*. Decamethonium modifies the initial rate of [^{3}H]α-toxin binding, the maximal amount of [^{3}H]α-toxin bound at equilibrium, and the rate of dissociation of [^{3}H]α-toxin from its membrane site. These three classes of effects are studied successively.

Effect of Cholinergic Agonists and Antagonists on Initial Rate of [^{3}H]α-Toxin Binding to Electrophorus and Torpedo Membrane Fragments

As discussed in the preceding paper (6), the rate of dissociation of the [^{3}H]α-toxin–membrane site complex is considerably slower than its rate of association. Our Millipore filtration assay thus constitutes a reliable technique for measuring initial velocities of [^{3}H]α-toxin binding with the required precision (Fig. 1). Usually samples were filtered every minute for 5 min immediately after [^{3}H]α-toxin had been mixed with the membrane fragments. Depending on the concentrations of toxin and receptor used, the first three or five points fell on a straight line, the slope of which gives a measure of the initial rate of [^{3}H]α-toxin binding.

Consequences of preliminary incubation of membrane fragments with cholinergic agents on initial rate of [^{3}H]α-toxin binding. Several experiments *in vivo* have shown for a number of excitable membranes that the amplitude of the permeability change due to cholinergic agonists varies with the time of exposure to the agonist. Such a "desensitization" effect might be accounted for by a decrease of affinity and/or number of active receptor sites. We have studied the effect of preliminary incubation of membrane fragments with two cholinergic agents (decamethonium and *d*-tubocurarine) on the initial rate of [^{3}H]α-toxin binding. Membrane fragments from *Electrophorus* were diluted in Ringer's solution in the presence or absence of cholinergic agent at the desired concentration for different periods of time. The reaction was started by adding [^{3}H]-α-toxin. In some experiments the membranes were added immediately to the mixture of [^{3}H]α-toxin and effector in Ringer's solution.

When the preliminary incubation with 0.75 μM decamethonium or 0.13 μM *d*-tubocurarine was varied from 0 to 10 min, the same reduction in the initial rate of [^{3}H]-α-toxin binding was observed. In another

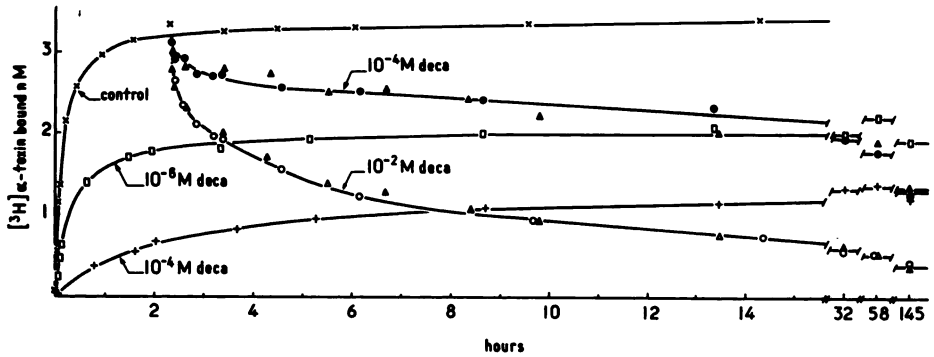

FIG. 1. *Effect of decamethonium on binding of [³H]α-toxin to membrane fragments from Electrophorus*
The time course of [³H]α-toxin binding to *Electrophorus* membrane fragments was followed by Millipore filtration as described under METHODS. The reaction medium contained 6.0 ± 0.3 nM active [³H]α-toxin and 0.57 mg of protein per milliliter. ×——×, binding of [³H]α-toxin in the absence of decamethonium (control); □——□ and +——+, membrane fragments were incubated for 10 min with decamethonium before the reaction was started by adding [³H]α-toxin; ●——● and ○——○, after 140 min a fraction of the control mixture was supplemented with 0.1 or 10 mM decamethonium by adding a small volume of concentrated decamethonium solution; ▲——▲ and △——△, same as above, except that decamethonium was added to the control mixture after 24 hr of incubation. These last curves were translated along the time axis to make them coincide with the reversal curves obtained after 140 min of incubation.

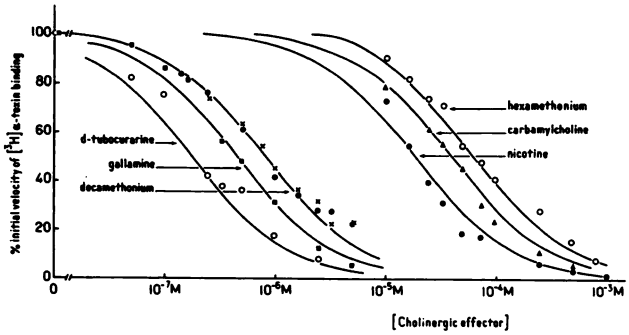

FIG. 2. *Effect of cholinergic agonists and antagonists on initial rate of [³H]α-toxin binding to membrane fragments from Electrophorus*
The curves were normalised to the initial rate measured in the absence of effector, and expressed as a percentage. The solid lines are theoretical hyperbolae having the same midpoint as the experimental curves.

experiment the membrane fragments were first incubated for 2 min with 15 μM decamethonium and then diluted 20-fold. The initial rates of [³H]α-toxin binding were measured 0 and 5 min after dilution. Again no difference was seen, and both sets of data were similar to those mentioned above. In all instances the initial velocity of [³H]-α-toxin binding was controlled by the actual concentration of decamethonium present at the time [³H]α-toxin was added, whatever the previous treatment of the membrane fragments. In other words, no "desensitization" was detected by this method at the level of the α-toxin–decamethonium binding site. The same experiments, moreover, indicate that establishment of the equilibrium of decamethonium with its binding site can be considered instantaneous in the time scale of our measurements. Nevertheless, to be certain, we routinely equilibrated the mem-

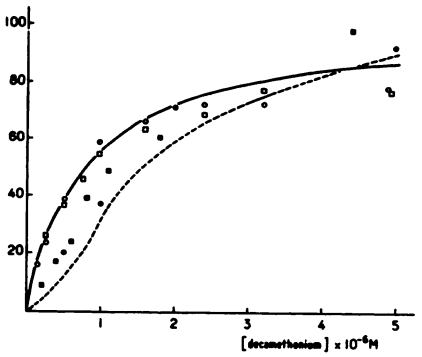

FIG. 3. *Comparison of curve for protection by decamethonium against [³H]α-toxin binding with curve of [¹⁴C]decamethonium binding to excitable membrane fragments from Electrophorus and dose-response curves measured in vivo and in vitro*

○ and □, percentage decrease of the initial rate of [³H]α-toxin binding as a function of increasing decamethonium concentrations. At saturating levels of decamethonium the initial rate becomes negligible (100 on the ordinate). The data are replotted from Fig. 2. ■, binding of [¹⁴C]decamethonium to excitable membrane fragments. The total number of [¹⁴C]decamethonium sites is taken as 55 nmoles/g of protein. The data are from Kasai and Changeux (5). ●, dose-response curve to decamethonium obtained *in vitro* by following $^{22}Na^+$ efflux from excitable microsacs; the data are from Kasai and Changeux (5). – – –, dose-response curve to decamethonium obtained *in vivo* with the isolated electroplax by following the steady-state membrane potential. The curve represents the average of five dose-response curves obtained with five different electric eels. The response curves have been normalised by assuming that 90% of the maximal depolarisation was achieved with 5 μM decamethonium. ——, theoretical curve (hyperbola) calculated from $F/(K_D + F)$, taking $K_D = 0.8$ μM for the dissociation constant of decamethonium.

brane fragments in Ringer's solution in the presence of cholinergic effector at its final concentration for 10 min before adding [³H]α-toxin.

Quantitative analysis of effects of decamethonium and d-tubocurarine on initial rate of [³H]α-toxin binding. Figure 2 shows that under the experimental conditions given in METHODS the initial rate of [³H]-α-toxin binding to *Electrophorus* membrane fragments decreases when the concentration of decamethonium increases, and becomes negligible at high concentrations of decamethonium. The curve of "protection" obtained follows a rectangular hyperbola up to at least 80% of its course (Figs. 2 and 3).

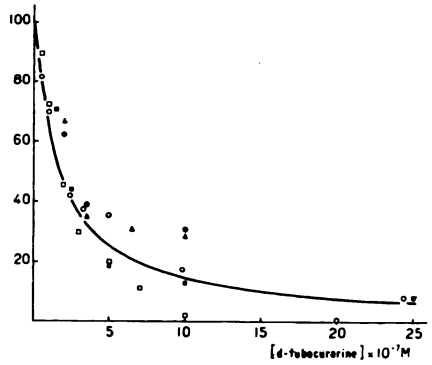

FIG. 4. *Curve of protection by d-tubocurarine against [³H]α-toxin binding; curve of displacement by d-tubocurarine of [¹⁴C]decamethonium bound to excitable membrane fragments from Electrophorus; and dose-response curves measured in vitro with excitable membrane fragments*

○, decrease in initial rate of [³H]α-toxin binding as a function of increasing *d*-tubocurarine concentrations. At saturating levels of *d*-tubocurarine the initial rate becomes negligible (0 on the ordinate). The data are replotted from Fig. 2. ● and ▲, displacement of [¹⁴C]decamethonium bound to excitable membrane fragments. The free concentration of decamethonium was 0.8 μM. The data are from Kasai and Changeux (5). Taking as g the fraction of sites occupied by [¹⁴C]decamethonium,

$$g = \frac{[\text{deca}]}{[\text{deca}] + K_{\text{deca}}\{(K_{\text{tubo}} + [d\text{-tubo}])/K_{\text{tubo}}\}}$$

where K_{deca} and K_{tubo} are the dissociation constants for decamethonium and *d*-tubocurarine, respectively, and the quantities in brackets are the concentrations of ligands. Then the fraction of the total concentration of sites which is not occupied by *d*-tubocurarine is

$$\frac{K_{\text{tubo}}}{K_{\text{tubo}} + [d\text{-tubo}]} = \frac{K_{\text{deca}}}{[\text{deca}]} \times \frac{g}{1 - g}$$

The value used for K_{deca} was 0.8 μM. ■ and □, antagonism by *d*-tubocurarine of the response *in vitro* of excitable membrane fragments to 100 μM carbamylcholine (■) or 0.8 μM decamethonium (□). The response was measured by following $^{22}Na^+$ efflux. The data are from Kasai and Changeux (5). Same plot as for binding data. ——, theoretical curve (hyperbola) calculated from $K_{\text{tubo}}/(K_{\text{tubo}} + [d\text{-tubo}])$, taking 170 nM as the dissociation constant of *d*-tubocurarine.

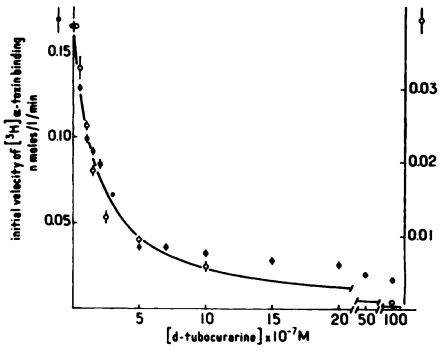

FIG. 5. *Effect of d-tubocurarine on initial rate of [³H]α-toxin binding to membrane fragments from Torpedo*

Membrane fragments from *Torpedo* prepared according to Cohen *et al.* (9) (0.75 mg of protein per milliliter, 475 nmoles of α-toxin binding sites per gram of protein) were diluted in *Torpedo* Ringer's solution supplemented with *d*-tubocurarine. After incubation for 1.5–4 hr at room temperature, the reaction was started by adding [³H]α-toxin (10.5 Ci/mmole, 58 nM). The kinetics of binding of the toxin was followed as described under METHODS. ●, 0.5 nM active [³H]α-toxin and 7.1 nM [³H]α-toxin binding sites; second-order rate constant, $k_1 = 4.55 \times 10^7$ M^{-1} min^{-1}. ○, 0.65 nM active [³H]α-toxin and 1.42 nM [³H]α-toxin binding sites; $k_1 = 4.25 \times 10^7$ M^{-1} min^{-1}.

The solid line is the theoretical hyperbola having the same mid-point as the experimental curves.

The same result is observed with *d*-tubocurarine and membrane fragments from both *Electrophorus* (Fig. 4) and *Torpedo* (Fig. 5).

In order to test whether the shape and position of the protection curve are characteristic of the cholinergic effector, experiments were performed at two different ratios of [³H]α-toxin to membrane sites. When normalized to the same maximal value, the two curves agree quite well (Fig. 5). Above 80% reduction of the initial rate, a small deviation was noticed at high concentrations of [³H]α-toxin and membrane fragments. The protection curve therefore seems characteristic of the interaction of the cholinergic ligand with its membrane site.

As shown in Figs. 4–6, the protection data are, to a first approximation, fitted by

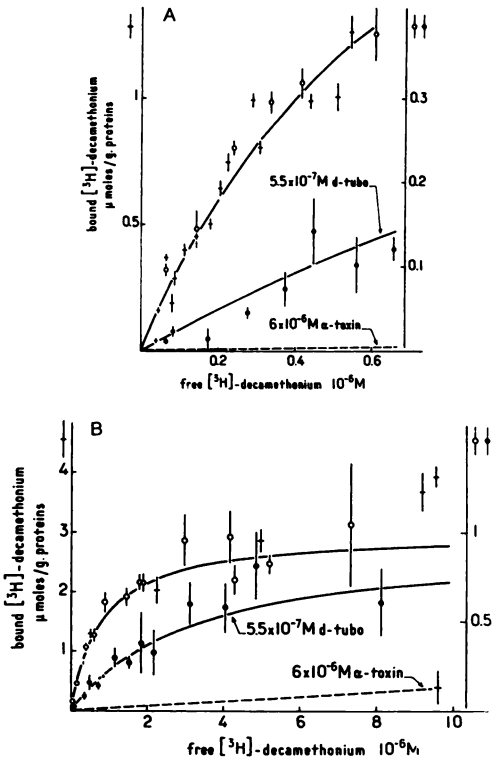

FIG. 6. *Binding of [³H]decamethonium to membrane fragments from Torpedo*

In experiment 1 (crossed lines) the membrane suspension contained 0.32 mg of protein per milliliter and 1.4 ± 0.2 μmoles of [³H]α-toxin binding sites per gram of protein. The concentration of [³H]decamethonium binding sites was 0.83 ± 0.3 μM (2.6 μmoles/g of protein). The dissociation constant was 0.74 ± 0.12 μM (see Fig. 8). When the total concentration of [³H]decamethonium was 1 μM, 9800 out of a total of 15,850 cpm/50 μl remained in solution after centrifugation; 38% of the counts were thus displaced. In experiment 2 (○ and ●) the protein concentration was 0.33 g/liter and the concentration of [³H]decamethonium binding sites was 0.33 ± 0.07 μM (1.0 ± 0.2 μmoles/g). The dissociation constant was 0.85 ± 0.15 μM. Points from experiments 1 and 2 were normalised to the same maximal value. Curves are theoretical hyperbolae calculated from the equation

$$[\text{deca}]_{\text{bound}} = B_{\max} \times \frac{[\text{deca}]_{\text{free}}}{[\text{deca}]_{\text{free}} + K_{\text{deca}}(1 + [\text{tubo}]/K_{\text{tubo}})}$$

with $K_{\text{deca}} = 0.8$ μM and $K_{\text{tubo}} = 0.17$ μM.

The experiments in Fig. 6A and Fig. 6B are identical except that a different range of decamethonium concentrations was explored.

TABLE 1

Comparison of protection constants (K_p) and equilibrium dissociation constants (K_D) with apparent dissociation constants measured in vivo and in vitro for a variety of cholinergic ligands on Electrophorus membrane fragments or electroplax

Data *in vivo* are from various authors [see references cited by Kasai and Changeux (5)], except for muscarone, acetylcholine, and nicotine. Data *in vitro* for $^{22}Na^+$ flux and binding of [^{14}C]decamethonium to microsacs are from Kasai and Changeux (5); data for binding of [^{3}H]decamethonium to the purified receptor protein in detergent solution (1% Triton X-100) are from Meunier and Changeux (10).

Effector	*In vitro*				*In vivo*: Membrane potential of isolated electroplax at 22°, K_{app}
	Protection against [^{3}H]α-toxin binding at 22°, K_p	[^{14}C]Decamethonium binding to microsacs at 22°, K_D	[^{3}H]Decamethonium binding to purified receptor protein in solution at 4°, K_D	$^{22}Na^+$ efflux from microsacs at 22°, K_{app}	
	M	*M*	*M*	*M*	*M*
Agonists					
Decamethonium	0.8×10^{-6}	1.3×10^{-6}	2.1×10^{-6}	1.2×10^{-6}	1.2×10^{-6}
Carbamylcholine	4.0×10^{-5}	2.2×10^{-5}	1.9×10^{-6}	4.0×10^{-5}	3.0×10^{-5}
Phenyltrimethylammonium			1.2×10^{-6}	2.0×10^{-5}	1.3×10^{-5}
Nicotine base	1.8×10^{-5}				$(2.3 \pm 0.5) \times 10^{-5}$
Muscarone	$(0.63 \pm 0.02) \times 10^{-5}$				$(1.6 \pm 0.3) \times 10^{-5}$
Acetylcholine[a]	$(1.5 \pm 0.2) \times 10^{-6}$				$(1.15 \pm 0.15) \times 10^{-6}$
Antagonists					
d-Tubocurarine	1.7×10^{-7}	2.0×10^{-7}	3.9×10^{-7}	1.5×10^{-7}	1.6×10^{-7}
Gallamine	4.4×10^{-7}	4.0×10^{-7}	1.3×10^{-7}	3.3×10^{-7}	3.0×10^{-7}
Hexamethonium	6.1×10^{-5}		6.2×10^{-5}	6.2×10^{-5}	3.0×10^{-5}

[a] In the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate.

the empirical equation

$$v_i = v_{i,0} \times \frac{K_p}{K_p + F}$$

where $v_{i,0}$ is the initial rate measured in the absence of effector, F is the concentration of effector, and K_p is a "protection" constant characteristic of the ligand. A measure of K_p is the concentration of effector for which $v_i = v_{i,0}/2$.

This result can be interpreted simply by assuming that (a) the association of [^{3}H]-α-toxin with its membrane site is slow compared to the rate of cholinergic ligand binding and (b) [^{3}H]α-toxin and F bind to the same class of membrane sites in a mutually exclusive manner. v_i is then proportional to the concentration of sites which are *not* occupied by F, and K_p becomes the actual dissociation constant of F from its membrane site in the absence of [^{3}H]α-toxin.

The first assumption had already been tested; in the time scale of the experiment (minutes), equilibration of the cholinergic ligand with its site is almost instantaneous and the dissociation of the toxin-membrane complex is negligible.

The second assumption was tested directly by measuring, with the same membrane preparation and the same ligands, protection constants (K_p) in the presence of [^{3}H]α-toxin and equilibrium dissociation constants (K_D) in the absence of [^{3}H]α-toxin.

Comparison of K_p and K_D for decamethonium and d-tubocurarine. Data on the direct binding of [^{14}C]decamethonium to

Electrophorus membrane fragments have been published by Kasai and Changeux (5). The same authors have also derived a value for the dissociation constant of *d*-tubocurarine from experiments in which [^{14}C]-decamethonium bound to the same membrane fragments was displaced by *d*-tubocurarine. In Figs. 3 and 4 and Table 1 the binding curves are compared with the protection curves obtained with the same ligands by following the decrease of the initial rate of [^{3}H]α-toxin binding: the agreement is excellent for both decamethonium (K_p = 0.8 μM, K_D = 1.3 μM) and *d*-tubocurarine (K_p = 0.17 μM, K_D = 0.2 μM). Within experimental error, under the present conditions of assay, K_p is almost identical with K_D. In other words, the curve of protection can be taken as a binding curve, and the constant of protection identified with the equilibrium dissociation constant of the cholinergic ligand.

We repeated the experiment with a membrane preparation in which direct binding could be measured more easily than with *Electrophorus* membrane fragments. We selected the membrane preparation from *Torpedo* recently developed by Cohen *et al.* (9). The specific activity of [^{3}H]α-toxin binding sites in this preparation is approximately 100 times higher than that of *Electrophorus*, and micromolar concentrations of sites become accessible in the test tube.

Direct binding to *Torpedo* membrane fragments of [^{3}H]decamethonium and [^{3}H]-acetylcholine in the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamine)ethyl phosphorothiolate was measured by centrifugation (see METHODS). The high concentration of binding sites in this preparation enabled us to measure binding with good accuracy (Fig. 6). Moreover, these *Torpedo* membrane fragments contained little acetylcholinesterase: approximately one catalytic site of acetylcholinesterase per 100 toxin binding sites (9). Binding of the cholinergic ligands to the catalytic site of acetylcholinesterase was expected to be negligible. Indeed, we found that gradual addition of unlabelled α-toxin led to complete displacement of [^{3}H]decamethonium and [^{3}H]-acetylcholine from these membrane fragments (Figs. 6 and 10) when the free concentrations of [^{3}H]decamethonium or [^{3}H]-acetylcholine were close to their K_D values.

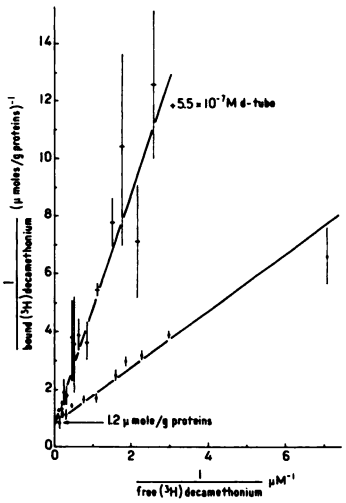

FIG. 7. *Competitive displacement by d-tubocurarine of [^{3}H]decamethonium bound to membrane fragments from Torpedo: double-reciprocal plot*

Data are from experiment 2 of Fig. 6. The total number of [^{3}H] decamethonium sites was estimated to be 1.0 ± 0.2 μmoles/g of protein. The dissociation constant for decamethonium was 0.85 ± 0.15 μM, and that for *d*-tubocurarine was 0.17 ± 0.05 μM.

The binding of [^{3}H]decamethonium and [^{3}H]acetylcholine also decreased in the presence of *d*-tubocurarine. For instance, 8 μM *d*-tubocurarine displaced 95 ± 5% of the counts bound in the presence of 0.8 μM free [^{3}H]decamethonium.

The double-reciprocal plot of the binding data for [^{3}H]decamethonium in the absence of *d*-tubocurarine (Fig. 7) does not deviate from a straight line within the precision of our experiment, up to a free concentration of decamethonium of 3 μM. Extrapolation of this line to the ordinate offers an estimate of the number of sites, 1000 ± 200 nmoles/g of protein. The number of [^{3}H]α-toxin binding sites in the same preparation was 1400 ± 200 nmoles/g of protein. The two values are thus close to each other. From the slope of the straight line, we calculated a dissociation constant for decamethonium of $K_{D,deca}$ = 0.85 ± 0.15 μM, a value rather close to that found with *Electrophorus* membrane fragments (5). In the same plot are shown the data obtained in the presence of

a total concentration of 0.55 μM *d*-tubocurarine. Again the data are adequately fitted by a straight line which intersects the ordinate at the same point as the line ob-

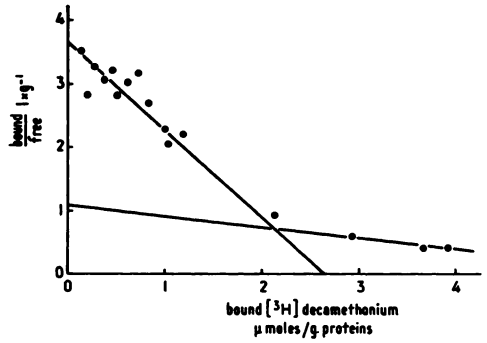

FIG. 8. *Scatchard plot of binding of [³H]decamethonium to membrane fragments from Torpedo*

Data are from experiment 1 of Fig. 6. The solid lines give a dissociation constant of 0.74 ± 0.12 μM and 2.6 μmoles/g of protein for the number of high-affinity binding sites, and 5.6 μM and 3.6 μmoles/g of protein for the low-affinity sites.

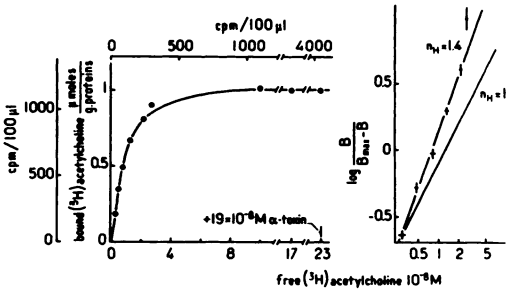

FIG. 9. *Binding of [³H]acetylcholine to membrane fragments from Torpedo*

Membrane fragments (320 ± 50 nM [³H]α-toxin binding sites; 0.6 mg of protein per milliliter) were diluted 3 times in a medium with the final ionic composition of *Torpedo* Ringer's solution. Binding of [³H]acetylcholine in the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate was measured by ultracentrifugation as described under METHODS. At the lowest concentration of [³H]acetylcholine used, the radioactivity before centrifugation was 258 cpm/0.1 ml; after centrifugation it was 43 cpm/0.1 ml. These values were corrected for the background of the scintillation counter: 6 ± 1 cpm. The right-hand graph shows a Hill plot of the binding data: $\log[B/(B_{max} - B)]$ as a function of log (free [³H] acetylcholine), where B is the concentration of bound [³H]acetylcholine and B_{max} is the total concentration of acetylcholine binding sites.

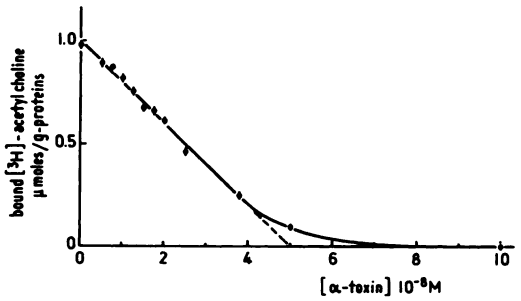

FIG. 10. *Displacement of [³H]acetylcholine bound to membrane fragments from Torpedo by N. nigricollis α-toxin*

Torpedo membrane fragments were diluted in *Torpedo* Ringer's solution containing 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate, to give a final concentration of [³H]α-toxin binding sites of 27 ± 3 nM. After incubation for 1 hr at room temperature with the desired concentration of α-toxin, [³H]acetylcholine binding was measured as described under METHODS. The total concentration of [³H]acetylcholine was 20 nM. In the absence of α-toxin, the concentration of free [³H]acetylcholine was 7.5 nM, which is very close to the value of the dissociation constant. The total number of [³H]acetylcholine binding sites was thus 25 nM (1059 nmoles/g of protein).

tained in the absence of *d*-tubocurarine. The antagonism between *d*-tubocurarine and decamethonium is thus competitive. The dissociation constant for *d*-tubocurarine is $K_{D\text{tubo}} = 0.17 \pm 0.05$ μM. The solid lines in Fig. 6A and B are the theoretical hyperbolae corresponding to the values of $K_{D,\text{deca}}$ and $K_{D,\text{tubo}}$ given above. When the free concentration of [³H]decamethonium becomes larger than 3 μM, binding of decamethonium to sites distinct from the previous ones was sometimes detected as shown on the Scatchard plot of Fig. 8. Although in this region of the curve the accuracy of our measurements is rather poor, we estimate that the dissociation constant of decamethonium from these sites is 5.6 μM and the number of sites is 3.6 μmoles/g of protein.

Since the affinity for [³H]acetylcholine is rather high (8 nM), its binding curve to *Torpedo* membrane fragments can be established with great accuracy. Figure 9 and Table 2 show a significant deviation from a rectangular hyperbola. The Hill plot of the

TABLE 2

Binding of [³H]acetylcholine to several preparations of membrane fragments from Torpedo

Protein	Concentration of [³H]α-toxin binding sites	Specific activity in [³H]α-toxin binding sites	Specific activity in [³H]acetylcholine binding sites	Dissociation constant for [³H]acetylcholine	n_H
g/l	*nM*	*nmoles/g protein*	*nmoles/g protein*	*nM*	
0.6	320 ± 50	530 ± 80	500 ± 10	8 ± 1	1.40
0.5	350 ± 50	700 ± 100	720 ± 80	10 ± 3	1.15
0.3	560 ± 60	1850 ± 200	1600 ± 200	8 ± 1	1.33
0.5	~750	~1500	1000 ± 100	8 ± 1	1.30
0.15	120 ± 20	800 ± 130	520 ± 40	7 ± 1	1.0
0.6	460 ± 50	770 ± 90	590 ± 40	10 ± 2	1.0

TABLE 3

Protection constants (K_p) and equilibrium dissociation constants (K_D) for a variety of cholinergic ligands and membrane fragments from Torpedo

Effector	K_p	K_D
	M	*M*
Acetylcholine[a]	$(8 \pm 1) \times 10^{-9}$	$(8 \pm 1) \times 10^{-9}$
Decamethonium	$(8 \pm 1) \times 10^{-7}$	$(7.4 \pm 1.2) \times 10^{-7}$
Carbamylcholine	$(5 \pm 2) \times 10^{-7}$	
Muscarone	$(3.8 \pm 1.3) \times 10^{-7}$	
Nicotine HCl	$(8 \pm 1) \times 10^{-7}$	
d-Tubocurarine	$(1.7 \pm 0.2) \times 10^{-7}$	$(1.7 \pm 0.5) \times 10^{-7}$
Hexamethonium	$(4 \pm 1) \times 10^{-5}$	
Choline	6×10^{-5}	2×10^{-4}
Atropine	1.5×10^{-4}	
Gallamine	8×10^{-6}	1×10^{-5}

[a] In the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate.

binding data gives a straight line, which, in four of six experiments, had a slope larger than 1 and close to 1.3. Cooperative effects in the binding of acetylcholine are present. Table 2 also reveals that the number of these sites completely blocked by the α-toxin (Fig. 10) does not differ significantly from the number of [³H]α-toxin sites estimated by the Millipore filtration method for the same preparation of membrane fragments.

As in the case of [³H]decamethonium, *d*-tubocurarine competitively inhibits the binding of [³H]acetylcholine. A double-reciprocal plot of the data gives, for the dissociation constant of *d*-tubocurarine, $K_{D,\text{tubo}} = 0.23 \pm 0.07$ μM. This value is in good agreement with that obtained from competition against [³H]decamethonium binding.

We next measured the effects of decamethonium, acetylcholine, and *d*-tubocurarine on the initial rate of [³H]α-toxin binding in the same membrane fragments. Table 3 shows that the constants of protection (K_p) measured by this method do not differ significantly from the dissociation constants (K_D) measured directly. This finding strengthens our former conclusion that the constants of protection K_p can indeed be taken as a measure of the equilibrium dissociation constants.

In the case of choline chloride, however, the protection constant $K_p = 60$ μM was found to be smaller than the dissociation constant $K_D = 200$ μM measured by displacement of [³H]acetylcholine binding. This discrepancy is still unexplained.

Comparative pharmacology of nicotinic receptors from Electrophorus and Torpedo electric organs. Protection against [³H]α-toxin binding by cholinergic ligands gives a convenient measure of equilibrium dissocia-

tion constants of these ligands. This method is more rapid than the classical methods of equilibrium dialysis and ultracentrifugation. Moreover, it does not require concentrations of binding sites of the same order of magnitude as the dissociation constant for the effector studied. We were thus able to estimate protection constants for cholinergic ligands of rather low affinity. We first investigated the binding of six nicotinic agonists and three nicotinic antagonists to *Electrophorus* membrane fragments. For each compound a protection curve was established (Fig. 2). The K_p values for these compounds are given in Table 1. In neighbouring columns of the same table we have reported the values for the "apparent" dissociation constants, determined either *in vitro* with excitable microsacs, by following $^{22}Na^+$ efflux, or in *vivo* with the isolated electroplax, by following steady-state depolarisation elicited by bath application of a cholinergic agonist. The quantitative agreement between "apparent" and "intrinsic" dissociation constants is particularly good. A slight deviation was noticed with the agonists, their K_{app} (*in vivo*) somewhat exceeding their K_p values.

To clarify this point, the complete dose-response curves and protection curves for decamethonium were plotted on the same graph (Fig. 3). The most evident difference was that the dose-response curve had a sigmoid shape while the protection and binding curves did not deviate significantly from rectangular hyperbolae. With the exception of this last point, the agreement between protection (binding) curves and dose-response curves was excellent for all the compounds tested, for agonists as well as antagonists (Fig. 4). In agreement with Kasai and Changeux (5), we confirm that with this system and with the method employed the dose-response curve for a given agonist can be superimposed on its binding curve. This correlation between the physiological response and the data *in vitro* leads us to conclude that the sites under study which bind both [^{3}H]α-toxin and nicotinic agents are indeed cholinergic receptor sites.

With *Torpedo* electroplax, dose-response curves are not yet available either *in vivo* or *in vitro*. However, there is no obvious reason why the methods and conclusions obtained with *Electrophorus* cannot be extended to *Torpedo*. It can be legitimately inferred with this system, as well, that the binding constants measured directly or by following the protection against [^{3}H]α-toxin binding are indeed characteristic of the "physiological" cholinergic receptor site.

As already mentioned, the K_p values for decamethonium and *d*-tubocurarine are approximately the same for *Torpedo* and *Electrophorus*. This correspondence was further explored by establishing the protection curves for eight more compounds. With all the other agonists (Table 3) [acetylcholine in the presence of *O*,*O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate, carbamylcholine, nicotine, and muscarone], the K_p values were as much as two orders of magnitude smaller than those found with *Electrophorus*. However, with one antagonist, gallamine, the affinity was smaller with *Torpedo* than with *Electrophorus*. The pharmacological properties of these two nicotinic receptors are thus strikingly different.

Effects of Cholinergic Agents on Binding at Equilibrium of [^{3}H]α-Toxin to Membrane Fragments from Electrophorus

The effects of cholinergic agonists and antagonists on the initial rate of [^{3}H]α-toxin binding can be accounted for simply on the basis of a mutual exclusion between cholinergic ligand and [^{3}H]α-toxin for the cholinergic receptor site. It was then of interest to test this conclusion at equilibrium. Equilibrium studies with [^{3}H]α-toxin as a ligand were made difficult because of the very slow rates of adsorption and desorption of the toxin. In particular, when the [^{3}H]α-toxin was added before the cholinergic ligands, equilibrium was not reached for several days. This is why in most of our experiments the system was equilibrated with the cholinergic ligand before adding the [^{3}H]α-toxin. Nevertheless identical plateau values were finally reached when toxin and cholinergic ligand were added in a different order but with the same final concentrations (Fig. 1).

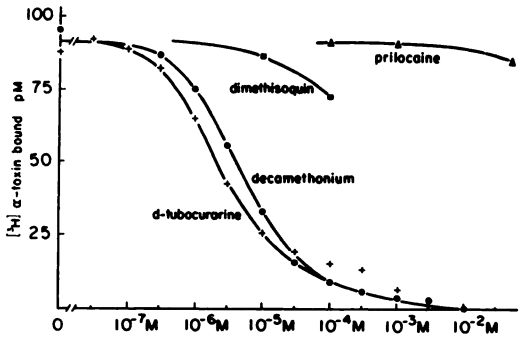

Fig. 11. *Effect of cholinergic effectors and local anaesthetics on binding at equilibrium of [³H]α-toxin to excitable membrane fragments from Electrophorus*

Membrane suspension (50 μl) was diluted in 20 ml of Ringer's solution supplemented with 0.02% NaN_3 and cholinergic effector or local anaesthetic. After a 10-min incubation the reaction was started by adding [³H]α-toxin. After overnight incubation at room temperature, the concentration of [³H]α-toxin bound was estimated by filtering a 5-ml sample as described under METHODS. Concentrations: binding sites, 180 pM; active [³H]α-toxin, 130 pM; protein, 0.026 g/liter. Under these conditions the time for half-completion of toxin binding in the absence of any effector was about 4 hr.

Figure 11 shows the effect of increasing concentrations of decamethonium and *d*-tubocurarine on the binding, at equilibrium, of [³H]α-toxin with 180 pM receptor sites and 130 pM [³H]α-toxin. It is clear that both ligands decrease the amount of toxin bound and that, at high concentrations of both, the displacement is almost complete.

The data can be analysed quantitatively on the basis of the assumption that decamethonium or *d*-tubocurarine competes with [³H]α-toxin for the same receptor site. The concentration α of [³H]α-toxin bound at equilibrium is then given by the equation

$$\alpha = a \frac{\beta}{\beta + K_\alpha(1 + F/K_D)} \qquad (1)$$

where a is the total concentration of binding sites, β is the concentration of free [³H]-α-toxin, and F is that of effector, K_α is the dissociation constant of the [³H]α-toxin, and K_D is that of the effector for the membrane site.

The logarithmic form of Eq. 1 is

$$\ln \beta \frac{(a - \alpha)}{\alpha} = \ln\left(1 + \frac{F}{K_D}\right) + \ln K_\alpha \qquad (2)$$

With Eq. 2 a plot of the experimental data, with $\ln \beta[(a - \alpha)/\alpha]$ as ordinate and $\ln (1 + F/K_D)$ as abscissa, should give a straight line of slope 1, which by extrapolation gives K_α when $F = 0$. Using for K_D the value determined from binding of the cholinergic ligands, we found that the equilibrium data can be fitted conveniently by Eq. 2; i.e., the data give a straight line of slope 1 up to 30 μM decamethonium and 10 μM *d*-tubocurarine (Fig. 12).

Above these values of ligand concentration a deviation appears. The significance of this deviation is not yet completely understood. In any case it cannot be accounted for by an increase of the rate of dissociation of the toxin (discussed in the following paragraph): the deviation would be in the opposite direction.

The dissociation constant K_α for the toxin, determined from the plot, is 20 ± 10 pM, a value remarkably close to that previously inferred from kinetic experi-

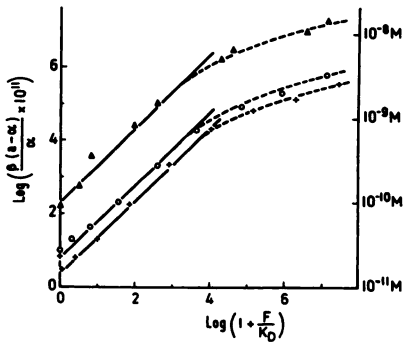

FIG. 12. *Logarithmic plot of effect of cholinergic effectors on binding at equilibrium of [³H]α-toxin to excitable membrane fragments from Electrophorus*

Data for the displacement of [³H]α-toxin binding by decamethonium (○——○) and *d*-tubocurarine (+——+) are replotted from Fig. 11. △——△, displacement of equilibrium by decamethonium in the presence of 2.8 nM [³H]α-toxin and 3.4 nM toxin binding sites. On both axes, the data are expressed in Napierian logarithms.

ments. Within a reasonable range of cholinergic ligand concentrations and at concentrations of toxin and receptor sites close to 100 pM, all the equilibrium data are thus consistent with the hypothesis of a mutual exclusion of the cholinergic ligands and [³H]α-toxin for the same class of sites.

We also performed a few equilibrium binding studies in the presence of nanomolar concentrations of [³H]α-toxin and receptor sites. For instance, one of the curves presented in Fig. 11 was obtained with 3.4 nM receptor sites and 2.8 nM [³H]α-toxin. Our log-log plot still yielded a straight line of slope 1, but the line was slightly displaced above the one obtained in the presence of lower concentrations of α-toxin. The reason for this phenomenon is yet not clear.

Effects of Cholinergic Agents on Rate of Dissociation of [³H]α-Toxin from Its Complex with Membrane Fragments from Electrophorous

In the preceding paper (6) it was shown that in the presence of an excess of unlabelled α-toxin, reversal of the binding of [³H]α-toxin to membrane fragments occurs with a half-time of 55 ± 5 hr.

The effect of the cholinergic effectors on both the initial rate of [³H]α-toxin binding and the amount of bound [³H]α-toxin at equilibrium are, in a given range of concentration, compatible with a simple model of mutual exclusion of α-toxin and cholinergic effector from a common binding site. It was thus expected that the kinetics of reversal of [³H]α-toxin binding would be the same in the presence of a large excess of α-toxin or cholinergic effector.

However, as shown in Fig. 1, the addition of high concentrations of decamethonium caused a rapid decrease in the amount of radioactivity retained on the Millipore filters. Figure 13 shows that this release of counts corresponds to the release from the membrane fragments of a molecular species with an exclusion volume on Sephadex G-50 column identical with that of free [³H]-α-toxin. Decamethonium thus enhances the apparent rate of dissociation of the α-toxin. This enhancement is the same after 2.5 or 24 hr of preliminary incubation of the membrane fragments with [³H]α-toxin (Fig. 1).

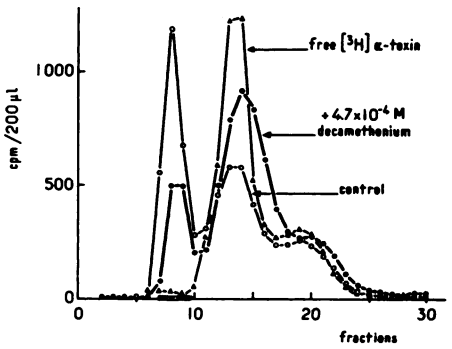

FIG. 13. *Characterisation of radioactive product displaced by decamethonium from Electrophorus membrane fragments labelled with [³H]α-toxin*

Membrane fragments from *Electrophorus* were incubated in Ringer's solution with [³H]α-toxin for 160 min at room temperature. Concentration of binding sites, 2.9 ± 0.2 nM; [³H]α-toxin, 5 nM. Half the reactive medium was then made 470 µM in decamethonium by adding a small volume of 0.1 M decamethonium. After 22 hr of incubation at room temperature, 200 µl of the mixtures were filtered through a 8 × 155 mm column of Sephadex G-50 (coarse) equilibrated with Ringer's solution. Fractions of 325 µl were collected at a flow rate of 8 ml/hr, and 200-µl samples were counted in 10 ml of Bray's solution. The column was calibrated with dextran blue (fractions 7–9) and with a small volume of the stock solution of [³H]α-toxin (14.8 Ci/mmole, 78% active).

One series of experiments was performed with concentrations of [³H]α-toxin and receptor sites lower than 500 pM. Figure 14A shows that the apparent rate of dissociation of the toxin–receptor site complex strongly depends on the concentration of decamethonium. The reversal becomes significant at concentrations higher than 100 µM. The half-maximal effect seems to occur at about 5 mM decamethonium, a concentration several orders of magnitude larger than the dissociation constant for the receptor site. In the presence of 10 mM and 100 mM decamethonium, complete release of the bound toxin occurs within a few hours. Figure 14B shows that the time course of the dissociation of 90–95% of bound α-toxin follows a single exponential. The remaining 5–10% is released rapidly within 15 min. The apparent rate of dissociation of the slow component depends on the temperature. At 20° the times for half-decay are 170 and

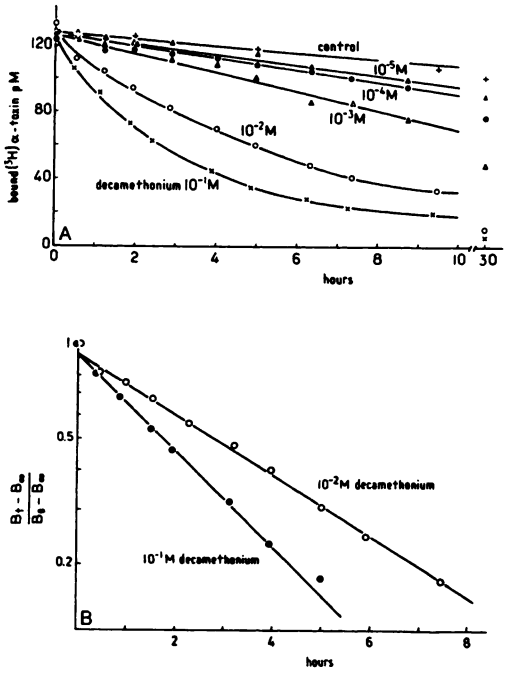

FIG. 14. *Effects of various concentrations of decamethonium on time course of [³H]α-toxin dissociation from Electrophorus membrane fragments*

Membrane fragments diluted in Ringer's solution were incubated for 39 hr at 20° with [³H]α-toxin (28 μg/ml of protein, 150 pM [³H]α-toxin binding sites, 200 pM active [³H]α-toxin). The medium was then supplemented with 1 M decamethonium. It was verified that increasing by 0.2 osM the ionic strength of the medium had no effect on the control kinetics. The slow decrease in bound [³H]α-toxin observed in the absence of decamethonium was probably due to an alteration of the membrane fragments after prolonged incubation. *A*. Arithmetic plot. *B*. Semilogarithmic plot of the same data. B_0 is the amount of [³H]α-toxin bound before addition of decamethonium. B_t is the amount bound at time t after addition of decamethonium. B_∞, the amount bound after 24 hr, did not differ from the background of the filters.

105 min in the presence of 10 and 100 mM decamethonium, respectively.

In the presence of 1 mM *d*-tubocurarine complete release also occurs, but the apparent rate of dissociation is considerably slower than in the presence of 10 mM decamethonium ($\tau_{1/2}$ = 650 min), although in both cases the concentrations used correspond to approximately 10^4 times their respective dissociation constants for the cholinergic receptor site. Both 10 mM gallamine and 120 mM carbamylcholine, concentrations which are again 10^4 times their K_D values, show only a small effect on the release: almost the same as 1 mM decamethonium. The order of efficiency of enhancing dissociation was decamethonium > *d*-tubocurarine > gallamine and carbamylcholine, while the affinities for the cholinergic receptor site followed the order *d*-tubocurarine and gallamine > decamethonium > carbamylcholine.

In another series of experiments, we studied the reversal of [³H]α-toxin binding at relatively high concentrations of receptor sites (2–3 nM) and of [³H]α-toxin. Figure 15 shows that under these conditions the kinetics of reversion is clearly biphasic: the time for half-decay of the slow component is not modified, but the proportion of [³H]α-toxin rapidly released in the presence of both 10 mM decamethonium and 2.5 mM *d*-tubocurarine becomes as large as 30% of the amount of [³H]α-toxin initially bound. In the presence of an excess of unlabelled α-toxin, the kinetics of reversion is also biphasic, but the slow component does not extrapolate at zero time to the value obtained in the presence of decamethonium and *d*-tubocurarine.

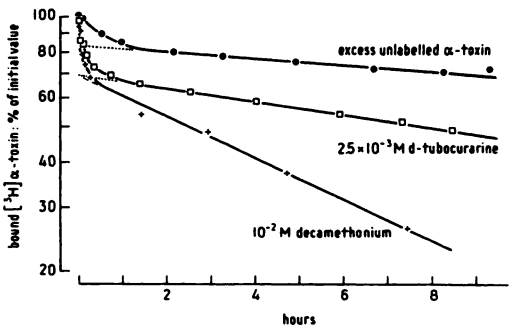

FIG. 15. *Biphasic dissociation of [³H]α-toxin bound to Electrophorus membrane fragments*

Membrane fragments were incubated overnight in Ringer's solution with [³H]α-toxin (0.23 mg/ml of protein, 3.7 nM [³H]α-toxin binding sites, 1.8 ± 0.2 nM active [³H]α-toxin). Reversal was started by adding a small volume of a concentrated solution of unlabelled α-toxin, *d*-tubocurarine, or decamethonium. The final concentration of unlabelled α-toxin was 1.5 μM.

TABLE 4

Effect of [³H]α-toxin concentration on slow and fast release of [³H]α-toxin from Electrophorus membrane fragments in the presence of 10 mM decamethonium

[³H]α-Toxin binding sites[a]	Active [³H]α-Toxin	Total [³H]α-toxin bound (*T*)	Slowly reversible [³H]α-toxin	Rapidly reversible [³H]α-toxin (*R*)	*R/T*
nM	nM	nM	nM	nM	%
2.1	0.24	0.160	0.135	0.025	15.5
2.1	2.4	1.74	1.22	0.52	30

[a] Millipore assay.

Moreover, as shown in Table 4, the percentage of rapidly released [³H]α-toxin depends, at a fixed concentration of binding sites, on the total concentration of [³H]α-toxin.

We then wondered whether the fact that the kinetics of dissociation seen in the presence of an excess of unlabelled α-toxin was slower than in the presence of decamethonium was not due to the presence of a diffusion barrier for α-toxin. This barrier, for instance, would deny access to the site of additional molecules of toxin, but not of decamethonium. To test this, labelled membrane fragments were solubilized with 1% Triton X-100 by the method of Meunier *et al.* (4). At a given time after the reversal was started by adding an excess of unlabelled α-toxin or 10 mM decamethonium, free and bound toxins were separated on a Sepharose 6B column. A parallel experiment was performed on membrane fragments by the usual Millipore filtration method. Figure 16 shows that the kinetics of reversal of [³H]α-toxin binding to solubilized receptor and to membrane fragments are consistent, and the same enhancement by decamethonium of the reversal rate was observed in both cases. This effect thus seems independent of the membrane environment of the cholinergic receptor.

DISCUSSION

We have defined experimental conditions under which [³H]α-toxin binding to excitable membrane fragments can be followed with good accuracy by simple filtration.

In a first series of experiments we followed initial rates of [³H]α-toxin binding to membrane fragments and confirmed that both cholinergic agonists and antagonists decrease them. Plots of the initial rate of [³H]α-toxin binding as a function of cholinergic effector concentration (protection curve) were fitted by rectangular hyperbolae, and complete protection occurred at high but still physiological concentrations of effector. All the data collected are, to a first approximation, compatible with the hypothesis that cholinergic effectors and [³H]α-toxin bind to a common membrane site in a mutually exclusive manner. It should be emphasized, however, that with *N. nigricollis* α-

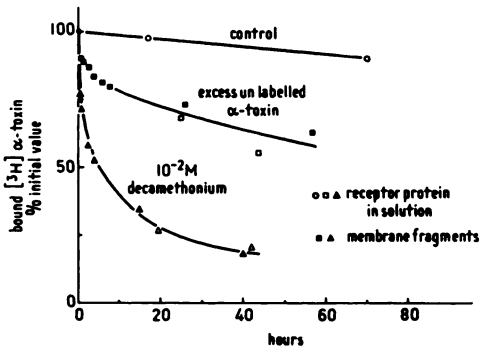

FIG. 16. *Effect of excess unlabelled α-toxin or decamethonium on dissociation of the receptor-[³H]α-toxin complex solubilised by Triton X-100*

Electrophorus membrane suspension (350 μl) was diluted in a 2.5-ml final volume of Ringer's solution. The concentrations of toxin binding sites and of active toxin were 7.8 and 5.0 nM respectively. After 2.5 hr of incubation at room temperature, the medium was diluted twice with 1 M Tris, pH 8.0, and 5% Triton X-100 and allowed to stand for 50 min at room temperature. The mixture was then centrifuged for 30 min at 100,000 × *g*. The pellet was discarded, and the supernatant fraction was made 1.5 μM in α-toxin or 10 mM in decamethonium. At given times 500-μl samples were filtered through a 9 × 205 mm Sepharose 6B column equilibrated with 0.1 M Tris, pH 8.0, 1% Triton X-100, and 0.02% NaN_3. Fractions of 500 μl were collected and counted. The amount of [³H]α-toxin bound was taken as the sum of the areas of the peaks of high molecular weight aggregates (V/V_t = 0.33) and of soluble receptor protein (V/V_t = 0.47). The free [³H]α-toxin was given by the area of the peak at V/V_t = 0.90, V_t being the total volume of the column.

toxin and with the membrane preparation we use, only a limited range of experimental conditions (α-toxin concentration, ratio of toxin to sites, etc.) can be explored. Deviation from strict competitivity between α-toxin and cholinergic ligands might occur (11) but has not been detected.

If the simple hypothesis of mutual exclusion for a common site is verified, the concentration of ligand which decreases by half the initial rate of [^{3}H]α-toxin binding (protection constant, K_p) should be identical with its equilibrium dissociation constant (K_D) measured directly in the absence of toxin. To test this point, we first compared our protection curves with the binding curves established directly with [^{14}C]decamethonium by Kasai and Changeux (5) on *Electrophorus* membrane fragments and found that for both decamethonium and *d*-tubocurarine these curves agree quite well. This result was confirmed with membrane fragments from *Torpedo*, which are particularly rich in toxin binding sites.

An extensive study of the binding at equilibrium of two radioactive cholinergic ligands, decamethonium and acetylcholine (in the presence of *O*,*O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate), was developed with *Torpedo* membrane fragments, using a simple centrifugation method analogous to that of O'Brien and Gilmour (12) and Kasai and Changeux (5). Binding data are consistent with the hypothesis that acetylcholine binds to a homogeneous population of slightly interacting (see below) sites with a dissociation constant K_D = 8 nM. On the other hand, decamethonium binding can be analysed in terms of two classes of independent sites of different affinities, of which only the high-affinity ones (K_D = 800 nM) are studied here. Interaction at equilibrium between acetylcholine or decamethonium and *d*-tubocurarine appears competitive. In addition, acetylcholine and decamethonium bound to their membrane sites are displaced by nearly, if not exactly, stoichiometric amounts of α-toxin. This was not the case in the previous work of Kasai and Changeux (5) with *Electrophorus;* in that case important nonspecific binding of decamethonium was noticed, in particular at the level of the catalytic site of acetylcholinesterase. Such contamination is considerably reduced with *Torpedo* membrane fragments, in which the number of catalytic sites is at least 100 times smaller than the number of toxin binding sites (6).

Nevertheless, with *Torpedo* membrane fragments, the numbers of α-toxin-sensitive sites for acetylcholine and decamethonium were very close, if not identical (with acetylcholine), to the number of [^{3}H]α-toxin binding sites measured by the filtration method described in the preceding paper (6).

Comparison of the direct binding curves with the protection curves showed that, as in the case of *Electrophorus* membrane fragments, the protection constants K_p for acetylcholine, decamethonium, and *d*-tubocurarine coincide quite well with their thermodynamic dissociation constants. Thus, with membrane fragments from both *Electrophorus* and *Torpedo*, the method of protection against [^{3}H]α-toxin binding gives an indirect but accurate measurement of the binding of the considered cholinergic effector.

The interaction of [^{3}H]α-toxin with its membrane site at equilibrium was also studied in the presence of cholinergic ligands. An excess of decamethonium or *d*-tubocurarine completely displaces [^{3}H]α-toxin from its binding sites. Again the results are, to a first approximation and within a range of concentration close to their dissociation constants for the cholinergic receptor site, consistent with a reversible and mutually exclusive toxin–cholinergic ligand interaction for a common binding site. Evidence for the presence of α-bungarotoxin binding sites which are not protected by *d*-tubocurarine (or only at very high concentrations) has been found by Miledi and Potter (13) and Chiu *et al.* (14) in crude extracts of frog sartorius and rat diaphragm, and by Albuquerque *et al.* (15) on end plates of mouse diaphragm. A similar result was obtained by Lester (16) following the binding of cobratoxin to frog end plates. The difference between these results and ours might be related to the fact that our starting material was always purified membrane fragments,

Complexity arose when the kinetics of dissociation of the [^{3}H]α-toxin from its membrane site was analysed. Indeed, since the data at equilibrium were accounted for by

the effect of cholinergic ligands on the association rate of [^{3}H]α-toxin binding, we did not expect any modification by these ligands of the rate of [^{3}H]α-toxin dissociation. In fact, decamethonium and *d*-tubocurarine, at concentrations several orders of magnitude larger than their dissociation constants for the cholinergic receptor site (measured in the absence of [^{3}H]α-toxin), strongly enhanced the release of [^{3}H]α-toxin from its complex with membrane fragments. Another characteristic of this effect is that the counts are released at two different rates at least: a fast one (minutes) for 5–30% of the radioactivity and a much slower one for the rest. While this phenomenon is not completely understood, it could result from structural heterogeneity of the toxin binding sites. All these sites, however, would bind both cholinergic ligands and [^{3}H]α-toxin and, according to our definition, should be considered cholinergic receptor sites. Another alternative is that release of the α-toxin follows a sequential mechanism. For instance, if, as already suggested (17, 18), the receptor protein is an oligomer, steric hindrance or other reasons might cause some of the toxin molecules bound to the same receptor oligomer to be more easily dissociated than others.

Finally, a slow but reversible transformation of the toxin-receptor complex might occur. Distinction among these various alternatives requires an extensive kinetic analysis and knowledge of the exact stoichiometry between the various classes of sites involved.

Enhancement of toxin dissociation by cholinergic ligands also cannot be simply interpreted. High concentrations of cholinergic ligands are required. Decamethonium enhances the dissociation more efficiently than *d*-tubocurarine, although the affinity of *d*-tubocurarine for the cholinergic receptor site is higher than that of decamethonium. This leads us to postulate that, in the concentration range explored, cholinergic ligands bind, once the α-toxin is bound, at sites at least partially distinct from the cholinergic receptor site in a strict sense. Khromov-Borisov and Michelson (19) have speculated that the cholinergic receptor site constitutes a complex surface with several anionic and esterophilic centers organised in a pattern able to fit multiquaternary ligands in a well-defined and characteristic manner. An interesting though untested hypothesis might be that, once bound, the α-toxin covers only a part of the pattern postulated by Khromov-Boriov and Michelson on the oligomeric protein and leaves some of its "subsites" accessible to cholinergic ligands. The binding properties of these subsites would no longer be those of the free receptor site.

The enhanced dissociation would be explained by either a steric or an allosteric effect of cholinergic ligand binding to these subsites by a mechanism which might present analogies with the enhanced deacylation of acetylcholinesterase by cholinergic ligand binding to a "peripheral" anionic center at least partially distinct from the catalytic center (20, 21).

Since at the present state of knowledge several alternative models can be proposed to account for the available experimental data, we have purposely avoided developing any particular one.

Under most of the experimental conditions *in vivo* and *in vitro* used throughout this work, the range of concentration of cholinergic ligands explored was such that binding to these "accessory" sites did not require consideration. Indeed, comparison of the dose-response curves obtained with *Electrophorus* electroplax or microsacs and of the protection or direct binding curves for decamethonium and *d*-tubocurarine shows clear-cut superimposition. The "apparent" dissociation constants are nearly identical with the intrinsic ones. Extension of this result to a large spectrum of cholinergic ligands further confirmed the early findings of Kasai and Changeux (5). Such a result may be surprising, since some of the dose-response curves were established from steady-state measurements of membrane potential upon bath application of the cholinergic ligand. There is no obvious reason why the changes of membrane potential should vary linearly with occupancy of the receptor sites by the agonist. It seems to be a favorable coincidence that with this system such an excellent correlation exists between response and binding.

The only difference—a minor but inter-

esting one— seen between measurements *in vivo* and *in vitro* concerns the shape of the titration curves. The dose-response curves generally were sigmoid, and this effect seemed less pronounced with the binding curves. In fact, a slightly sigmoid shape was repeatedly observed with acetylcholine [in the presence of *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate] on *Torpedo* membrane fragments. With decamethonium a similar deviation, if present, would remain undetected, being under the limits of resolution of our binding measurements. The Hill coefficient for acetylcholine was 1.3, a value significantly smaller than that measured *in vivo* on *Electrophorus* electroplax. The reason for this difference is not clearly understood. For instance, isolation of the membrane fragments might alter cooperative assembly of the cholinergic receptors.

By affinity chromatography the receptor protein from *Electrophorus* can be easily purified (22). As shown in Table 1, the affinities of three agonists for the purified protein in solution appear several orders of magnitude larger than those of the same agonists for the receptor protein present in membrane fragments. The affinity for the antagonists remains the same. One of the interpretations proposed by Meunier and Changeux (10) for this effect is a release of membrane constraint upon solubilisation and purification of the receptor protein.

The values of the dissociation constants

TABLE 5

Comparative survey of dissociation constants and protection constants for cholinergic ligands with membrane fragments from Electrophorus and Torpedo

Effector	*Electrophorus*		*Torpedo*		Franklin and Potter (28)
	Present work	O'Brien and co-workers (23, 24)	Present work	O'Brien and co-workers (24–27)	
	M	*M*	*M*	*M*	*M*
Acetylcholine[a]			8×10^{-9}	8×10^{-9} [100] 6.8×10^{-9} [830]	
Carbamylcholine	3×10^{-5}		5×10^{-7}		5×10^{-6}
Muscarone	6×10^{-5}	$3.6\text{–}5.5 \times 10^{-8}$ [15–20]		$2.2\text{–}6.5 \times 10^{-8}$ [60–80]	
			3.8×10^{-7}	$2.7\text{–}7.2 \times 10^{-7}$ [390–1000]	
Nicotine		$4.6\text{–}6.3 \times 10^{-8}$ [27–33]		2×10^{-7} [60–100]	
	1.8×10^{-5}	1.6×10^{-5} [293]	8×10^{-7}	$2.5\text{–}3.3 \times 10^{-6}$ [750–1300]	
Decamethonium		$2.5\text{–}3.2 \times 10^{-9}$ [30–36]		$0.7\text{–}1.3 \times 10^{-7}$ [60–500]	
		$1.9\text{–}55 \times 10^{-8}$ [20]	8.0×10^{-7}	$4\text{–}5.9 \times 10^{-7}$ [1400–2600]	
	8.0×10^{-7}	$8.3\text{–}25 \times 10^{-7}$ [250–293]		$0.8\text{–}2 \times 10^{-5}$ [3200–7400]	
		$5.2\text{–}10 \times 10^{-5}$ [3000–4000]			
d-Tubocurarine	1.7×10^{-7}		1.7×10^{-7}		5×10^{-6}

[a] In the presence of 0.1 mM *O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate. The number of binding sites in n moles per kg of fresh tissue is given in brackets. In the present work these numbers are approximately [100] for *Electrophorus* and [1000] for *Torpedo*.

for cholinergic ligands that we obtained with purified membrane fragments have been compared with those presented by various authors with cruder fractions of electric tissue.

In Table 5 are collected our results on *Electrophorus* and those of O'Brien and coworkers (23, 24). For nicotine and decamethonium, the sites we studied appear analogous to the low-affinity sites of O'Brien *et al.* With muscarone a large difference exists, which might be related to the fact that these authors did not study muscarone binding in the same range of concentration as we did. The strong correlation we observed between "apparent" and "intrinsic" dissociation constants, coupled with the fact that the α-toxin blocks the binding of cholinergic ligands, strongly supports the view that the sites we studied are indeed involved in the electrogenic action of acetylcholine.

Surprisingly, we have no evidence for the high-affinity sites found by O'Brien and coworkers for nicotine, muscarone, and decamethonium. However, the binding experiments of Kasai and Changeux (5) and our protection experiments might not have been accurate enough to detect these sites, since they are expected to represent only a small fraction of the total binding sites. Another explanation, already discussed by Eldefrawi *et al.* (25), is that the different classes of sites might be carried by different conformations of the same molecule. Our fractionation procedure may favor one of these conformations. Finally, we cannot exclude the possibility that some classes of sites were lost during fractionation.

Table 5 shows that a similar comparison can be made with *Torpedo*. For acetylcholine the high-affinity sites we studied coincide quite nicely with the high-affinity sites of Eldefrawi *et al.* (25) with respect to the dissociation constant (8 nM). However, Fig. 10 clearly indicates that no class of binding sites with a dissociation constant close to 68 nM existed in our preparation. Again we cannot exclude the hypothesis that these sites exist in the electric organ, but that the fractionation method of Cohen *et al.* (9) selects a given class of membrane fragments.

Franklin and Potter (28) have measured protection constants for four cholinergic effectors by the same method as ours, but with a total homogenate of *Torpedo* electric tissue. Although the results coincide quite well in the case of atropine and hexamethonium, there exists a large discrepancy with *d*-tubocurarine and carbamylcholine (Table 5). It might be due to the difference in ionic composition of the incubation medium or to heterogeneity of the binding sites in a total homogenate. In that case the protection constant may reflect a mean value of the dissociation constants for the different binding sites.

Unexpectedly, some of the values obtained with *Torpedo* differ strikingly from those reported with *Electrophorus*. Affinities for decamethonium and *d*-tubocurarine are approximately the same; however, the affinities of carbamylcholine, muscarone, and nicotine are approximately two orders of magnitude higher with *Torpedo* than with *Electrophorus*. The reasons for this difference are not clear, although it should be emphasized that *Electrophorus* and *Torpedo* belong to two different zoological orders, and the evolutionary distance between the two species might be much wider than between *Electrophorus* and mammals. Indeed, Patrick *et al.* (29) have obtained with mammalian muscle cells in culture values of dissociation constants for a few cholinergic effectors much closer to those of *Electrophorus* than those of *Torpedo*.

Moreover, in *Torpedo* the membrane fragments come from subsynaptic areas while in *Electrophorus* an important fraction of these fragments is derived from extrasynaptic ones. Although we still have no evidence for different properties between extrasynaptic and subsynaptic receptors, this possibility cannot be excluded.

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