STUDIES OF THE BINDING OF α-BUNGAROTOXIN TO MEMBRANE-BOUND AND DETERGENT-DISPERSED ACETYLCHOLINE RECEPTORS FROM *TORPEDO* ELECTRIC TISSUE

G.I. FRANKLIN and L.T. POTTER
Department of Biophysics, University College London, London WC1E 6BT, England

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1. Introduction

In order to study neuroreceptors directly in an impure state it is necessary to measure their interaction with a ligand. To date the greatest success has been achieved with the nicotinic acetylcholine receptors of vertebrate skeletal muscles [1–3] and of the electric tissues of the eel, *Electrophorus electricus* [4], and the fish, *Torpedo* [5,6], because of the availability of several neurotoxins from Elapid snakes which have been found to bind with very high affinity and nearly total specificity and irreversibility to these receptors. The toxin-binding substances in these tissues are membrane-bound proteins which appear to be similar if not identical (Molinoff and Potter, unpublished observations, cf. [7]). *Torpedo* electric tissue binds 1 μmole (8 mg) of α-bungarotoxin, the principal neurotoxin of the Formosan banded krait, *Bungarus multicinctus* [1], per kg of fresh tissue [5,7], and the principal receptor polymer recovered in solution after detergent-dispersion of the membrane proteins from this tissue [7] as well as eel electric tissue [4,8] appears to be a hexamer composed of subunits of 42,000 daltons.

The function of nicotinic receptors is to couple the arrival of acetylcholine at post-synaptic membranes with the opening of ion channels through the membranes, and acetylcholine probably produces a conformational change in receptors which initiates this response [9,10]. Evidence that desensitization of receptors in muscles with acetylcholine or carbamylcholine causes a slowing of binding of Elapid neurotoxins [2, 11], but an acceleration of binding of an alkylating derivative of decamethonium [10], indicates that these receptors can assume two different conformational states. Since conformational changes of a protein may be different when it is membrane-bound compared to when it is in solution, and since some detailed studies of nicotinic receptors require their isolation from membranes, it becomes important to know what sort of conformational changes, if any, persist in solution. As one approach to this question we have begun to study the effects of several drugs on the rate of binding of α-bungarotoxin to receptors in membranes and in solution, with the idea in mind that an agonist like carbamylcholine might produce different effects in the two environments. The results show that the rate of binding of this toxin is unaltered in solution and that the antagonist, d-tubocurarine, has the same effect in slowing binding in the two conditions. However ten times as much carbamylcholine is required to halve the binding rate to receptors in solution as is required with membrane suspensions. Compounds which are known to have little effect on receptors *in situ*, as determined by physiological measurements, had little effect on toxin-binding in solution at the usual physiological doses, and a compound which accelerates desensitization and blocks the receptor-response mechanism in muscles was also shown to have little effect in toxin–receptor interactions.

2. Materials and methods

Torpedo marmorata about 30 cm across were ob-

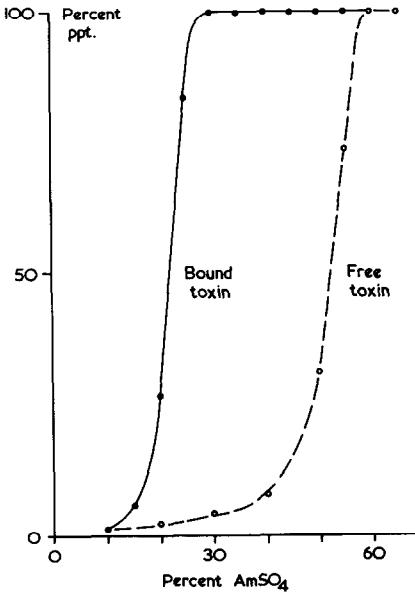

Fig. 1. Precipitation of free and membrane-bound α-bungarotoxin by ammonium sulfate. For the free toxin assays about 0.1 μg of toxin in 1 ml of the solution used to dilute detergent-dispersed membrane proteins was mixed with 10 ml of sufficient ammonium sulfate in 0.2 M citrate–phosphate buffer, pH 5, to achieve the final percent-saturation levels shown on the abscissa. The assays for bound toxin were conducted in the same manner, except for the presence of toxin-saturated receptors instead of free toxin; to prepare toxin–receptor complexes, toxin (20 μg/g tissue) was added to the membranes which had been isolated and dissolved as described in the text. The percent of the total radioactivity which was precipitated and collected on the Millipore filters is shown on the ordinate. The points in this figure (and in subsequent figures unless otherwise stated) are the results of single filtrations. Note that precipitated toxin–receptor complexes are not redissolved or dissociated at high concentrations of ammonium sulfate

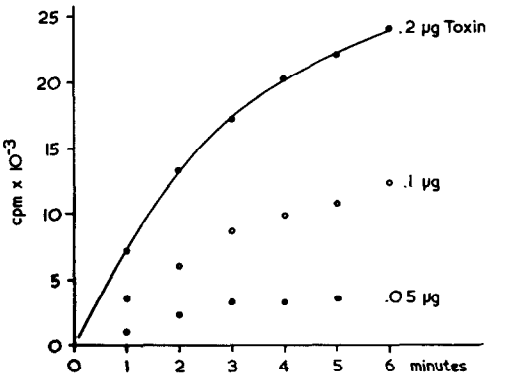

Fig. 2. Binding of α-bungarotoxin to membrane fragments. The amounts of toxin indicated were used to start the binding reaction described in the text. The solid line was calculated from the k_1 value determined from the associated points.

tained through the courtesy of Dr. R. Martin and the staff of the Zoological Station, Naples, Italy. The fish were transported, the electric organs were dissected, and washed membranes were prepared by slight modifications of previously described procedures [5, 12]. Briefly, the skin was stripped from frozen tissue, and 100 g was homogenized in a Waring blender for 3 × 1 min (with 2 min intervals on ice) in 300 g of ice-cold 20 mM sodium phosphate buffer, pH 7.4, containing 0.02% NaN_3 ("buffer"). The homogenate was poured through a nylon sieve having holes 1 mm square, to remove large shreds of connective tissue, and 40 g aliquots were centrifuged in weighed plastic tubes at 38,000 g_{max} for 1 hr to fully precipitate membrane fragments and osmotically-lysed subcellular particles. The supernatant fluid was aspirated and discarded, and the pellets were frozen until required. For use a pellet was unfrozen, buffer was added to a final weight of 20 g, and the particles were resuspended with a Polytron blender equipped with a P10 generator and operating at 22,000 rpm for 1 min. This suspension was poured through 25 μm nylon mesh before use, yielding particles from 1 g of tissue and having 10–12 mg of protein per 2 g of suspension. Membrane proteins were dissolved by adding 0.1 vol of 10% Triton X-100 (w/w) to this suspension. The mixture was left for 1 hr on ice and was then centrifuged at 38,000 g_{max} for 30 min; buffer was added to the clear supernatant fluid if necessary so that dissolved membrane proteins from 1 g of tissue (8–10 mg) were present in 2 g of fluid. These preparations of membrane material were kept at 4° and were usually used within 2–3 weeks; variation in the toxin binding rate only being observed in the third week.

Alpha-bungarotoxin was prepared and labelled with ^{131} iodine as previously described; the initial specific activity was 20–30 thousand Ci/mole [12]. Protein was assayed by a micro Folin-reagent procedure [13].

Binding of the toxin to membrane-bound receptors was assessed as follows. One half ml of resuspended

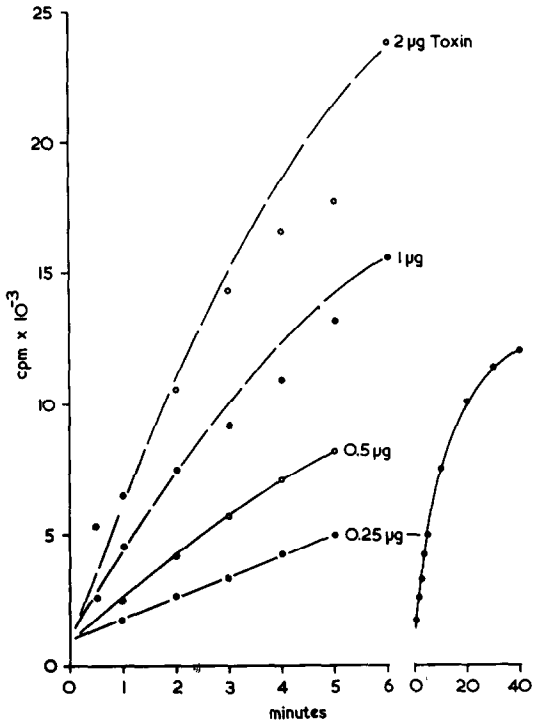

Fig. 3. Binding of α-bungarotoxin to Triton-dispersed membrane proteins. The amounts of toxin indicated were used to start the binding reaction described in the text. The solid line (0.5 μg of toxin) was calculated from the k_1 value determined from these results. Since the reaction rate was nearly constant for 5 min with 0.25 μg of toxin, this level of toxin was often used for subsequent experiments, although the concentration was also varied between 0.05 and 2 μg. The zero-time value indicated by extrapolation of the results (dashed line) was greater than the free toxin blank value obtained without membrane proteins, which has been subtracted.

membranes from 0.25 g of tissue was diluted with 9.45 ml of ice-cold buffer to yield a dilute suspension capable of binding about 2 μg or 2.5×10^{-10} moles of α-bungarotoxin [5]. Where indicated, various drugs were included in the buffer and the particles were left for 5 min before the addition of α-toxin. To start the binding reaction, 50 μl of buffer containing 0.05–20 μg of labelled toxin were rapidly mixed into the particle suspension. At 1 min intervals thereafter, 0.5 ml aliquots were rapidly diluted into 10 ml of buffered 0.4 M NaCl containing 0.25 mg/ml bovine serum albumin (BSA), and within 30 sec the fluid was filtered on 2.5 cm diameter filters of mixed esters of cellulose having an average pore diameter of 0.45 μm (Millipore, HAWP). Salt and BSA were included to minimize non-specific adsorption of the toxin to the filters. The filters were rinsed with 10 ml of the same buffered salt solution and were placed in 10 ml of a solution containing 9 ml toluene, 0.5 ml ethanol, 0.5 ml methanol and 4 mg diphenyloxazole for liquid scintillation spectrometry. A similar binding assay has been used by others to study the kinetics of binding of a radioactive cobra toxin to membrane fragments from eel electric tissue [14].

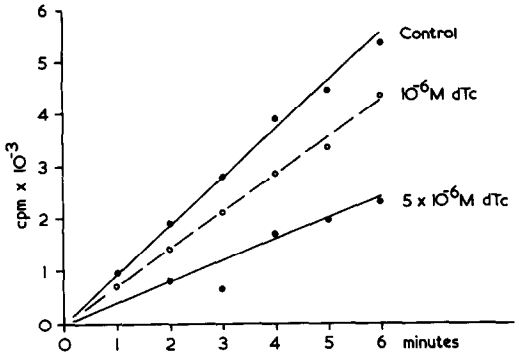

Fig. 4. Inhibition of toxin-binding to detergent-dispersed receptors by d-tubocurarine (dTc). In this and the subsequent figures each of the control points is the average of 2 determinations. The lines in this and last figure were drawn by eye, and the extrapolated blank value has been subtracted.

The binding of α-toxin to dissolved receptors was measured as follows. One half ml of Triton-dispersed membrane proteins from 0.25 g of tissue was diluted with 9.45 ml of ice-cold buffer containing 2 mg/ml BSA, 0.3 mg/ml bovine gamma globulin, 0.1 M NaCl, 0.1% Triton X-100 (w/w) and the drugs indicated in the text. For these assays BSA and gamma globulin served to co-precipitate toxin–receptor complexes in the presence of ammonium sulfate. Since Triton fully dissolves toxin-binding sites from *Torpedo* membranes [5,7], their concentration was about 2.5×10^{-8} M. After 5 min drug exposure, the binding reaction was begun by the addition of α-toxin as with membranes, but the separation of free from bound toxin was different. At 1 min intervals 1 ml aliquots of the reaction mixture were removed and mixed with 10 ml of 33%-saturated ammonium sulfate in 0.2 M citrate-phosphate buffer, pH 5, and after 1 min the faintly turbid fluid was filtered on Millipore discs as above in about 15 sec, and the filters were then washed with 10 ml of

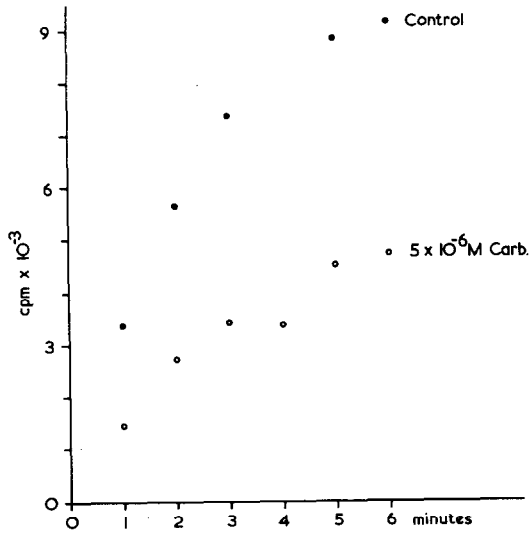

Fig. 5. Inhibition of toxin-binding to membrane-bound receptors by carbamylcholine (Carb.). The toxin concentration was 0.25 μg.

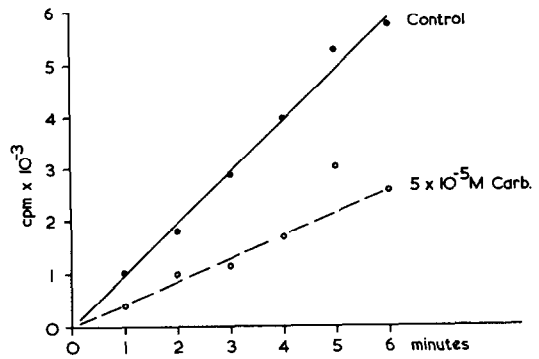

Fig. 6. Inhibition of toxin-binding to receptors in solution by carbamylcholine. In this and in fig. 4, the toxin concentration was 2 μg.

30%-saturated ammonium sulfate in the same buffer. This pH was used because it is the approximate isoelectric point of the predominant toxin–receptor polymer of *Torpedo* tissue [6,7]. A final concentration of 30%-saturated ammonium sulfate was found to fully precipitate toxin–receptor complexes while giving a reasonably minor precipitate of free toxin (fig. 1). In every experiment a sample of free toxin was filtered under the conditions used for the experiment, except for the presence of membrane material, and in most cases the "blank" value obtained was subtracted from the results given. For unexplained reasons, the blank values indicated by extrapolation of toxin-binding to time zero, were usually slightly higher than the free toxin blank value (e.g. fig. 2), and for some drug studies the "extrapolated blank" value indicated by the results was subtracted as indicated in the figure legends. In all experiments amounts of toxin required for subsaturation (about 1 μg/g tissue) and for full saturation of binding sites (20 μg/g tissue, at least 2-fold excess) were incubated for 8 hr with the membranes or membrane proteins and the samples were assayed to determine the percentage of the toxin which was active in binding (about 65%) and the total toxin-binding capacity of the preparation, both in terms of cpm. The initial concentration of active toxin (a in equation 1) and the initial concentration of binding sites (b) were determined from these results and from the specific activity of the stock solution for every experiment. It was assumed that the specific activities of active and inactive toxin were equal.

For the reaction: α-toxin + receptor $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ complex k_2 was assumed to be many orders of magnitude smaller than k_1, and the latter was calculated from the equation:

$$k_1 = \frac{2.303}{t(a-b)} \log_{10} \frac{b(a-x)}{a(b-x)} \qquad (1)$$

where x is the amount of bound toxin after time t.

3. Results and discussion

Typical results for the binding of α-toxin to membrane-bound and Triton-dispersed receptors are given in figs. 2 and 3, respectively. Average k_1 values (± SEM) were $2.0 \pm 0.4 \times 10^7$ M^{-1} min^{-1} (n=7) and $1.9 \pm 0.8 \times 10^7$ M^{-1} min^{-1} (n=9), respectively. These values correlate well with that (1.7×10^{-7} M^{-1} min^{-1}) found by Weber et al. for the binding of a neurotoxin from the cobra, *Naja nigricollis*, to membrane fragments from eel electric tissue in sucrose solution at 22°. In each case binding proceeded as a simple bimolecular reaction, indicating a single reactive form of receptors. Clearly the dissolution of receptors does not significantly impede binding of the toxin, and since the interaction of these proteins presumably involves a considerable part

of the surface of the receptor subunits, the present results may be taken as evidence that the conformation of the reactive part of receptors is little altered in solution. It is perhaps surprising that there is so little effect of the detergent on binding. Either detergent micelles around the toxin receptor do not have much effect on the approach of the two proteins, or the detergent binds primarily to nonreactive parts of the molecule, e.g. to the hydrophobic part of the receptor molecule which is normally imbedded in the post-synaptic membrane.

d-Tubocurarine is a classic competitive antagonist for nicotinic receptors, and at a concentration of $5 \times \times 10^{-6}$ M it approximately halved the rate of toxin-binding both to soluble (fig. 4) and to membrane-bound receptors. In each case a concentration of $2 \times \times 10^{-4}$ M was necessary to reduce binding to negligible levels. These results indicate that curare as well as the toxin has easy access to the reactive region of Triton-dissolved receptors, and show in addition that the part of the active site of receptors which is affected by curare is little altered in solution. From classic occupation theory a halving of the initial binding rate should occur when half the receptors are occupied by curare, and the concentration required should equal the dissociation constant for this drug. Accurate physiological values for the dissociation constant for curare and *Torpedo* tissue are not available because of the very slow diffusion of large molecules into the synaptic clefts of this electric tissue [5]; however the K value for d-tubocurarine for depolarization of eel electroplaques, and the calculated K for the effect of curare in slowing binding of a neurotoxin to eel membrane fragments are both 1.7×10^{-7} M [14]. The reason for the considerable difference between the values for eel and *Torpedo* electric tissue is not yet apparent.

Carbamylcholine was used as an agonist instead of acetylcholine because it is less readily hydrolysed by acetylcholinesterase. With membrane-bound receptors k_1 was reduced by approx. half with 5×10^{-6} M carbachol (e.g. fig 5), whereas 5×10^{-5} M was required for the same effect in solution (fig 6). The reason for this difference is not clear. In view of the results with α-bungarotoxin and curare, the difference is not easily attributed to a different rate of access of carbachol to active sites of receptors on membranes and in solution; however further experiments with other drugs will be necessary to settle this point. Assuming for the sake of argument that there is a change in the effects of agonists on receptors in solution, as measured by a change in binding of a neurotoxin, there are at least five possible explanations for the change: i) There is the possibility that α-bungarotoxin binds equally well to receptors in resting and agonist-altered states as a consequence of dissolving membrane proteins. This alternative will be examined by studying the rate of binding of an alkylating derivative of decamethonium which reacts most readily with desensitized receptors [10]. ii) The possibility that no conformational change occurs in solution seems unlikely, although the present results could be explained easily if the action of an agonist in solution were only due to site occupation (competition) while the effect on membranes was due both to competition and to reversible changes in receptor conformation. Again, studies with other drugs may resolve this question. iii) The possibility of an irreversible or only slowly reversible conformational change in receptors in solution is unlikely, since carbachol should then have been more, rather than less potent, in solution. iv) The simplest explanation for the results is that there is a true change in the affinity of the recognition sites for carbachol, despite the lack of change for curare. v) A more appealing alternative is that the molecules in solution "desensitize" i.e. assume an altered conformation which binds toxin at a slower rate, for a shorter period. Again there are no accurate physiological values for dissociation constants for carbachol in intact *Torpedo* tissue; the value for eel tissue is about 3×10^{-5} M.

Atropine is an excellent competitive antagonist at muscarinic acetylcholine receptors, having a dissociation constant of about 10^{-9} M in most preparations, but it has little effect on nicotinic receptors except at concentrations above 10^{-4} M. When tested on toxin-binding in solution it produced no inhibition until this concentration was reached, and 50% inhibition appeared to require $5-10 \times 10^{-4}$ M. Neostigmine, which is a potent inhibitor of acetylcholinesterase at 10^{-6} M, has similar effects on receptors *in situ* as atropine; it reduced k_1 by half only at a concentration of 10^{-3} M. Hexamethonium, which blocks acetylcholine receptors in ganglia better than in muscles, was more potent, producing some inhibition at 10^{-5} M and 50% inhibition in the range $5-10 \times 10^{-5}$ M. This result correlates well with the effect of hexamethonium on the depolarization of eel electroplaques (3×10^{-5} M) and slow-

ing of binding of a toxin to eel electric tissue membranes (6×10^{-5} M). Tetrodotoxin, which fully blocks the active sodium channel of action potentials at $6 \times \times 10^{-6}$ M was not expected to, and did not have, any effect on toxin-binding in solution at this concentration.

Finally an interesting result was obtained with SKF 525A, the diethylaminoethyl ester of diphenylpropylacetic acid. Under certain circumstances this compound can be as effective as curare in blocking the responses of skeletal muscle to depolarizing agents, although different mechanisms seem to be involved. The available evidence suggests that SKF 525A accelerates desensitization by acting on the response mechanism at a stage later than receptor activation [15]. When tested for its effect on toxin-binding in solution, k_1 was reduced by half only with at least 10^{-3} M and no effect was seen at 10^{-4} M. These results support the postulate that this antagonist affects receptor-linked responses without reacting significantly with the part of receptors which is affected by acetylcholine, curare, and α-bungarotoxin.

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