α-Bungarotoxin Binding and Cholinergic Receptor Function on a Rat Sympathetic Nerve Line*

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A clonal rat sympathetic nerve cell line, PC12, binds iodinated α-bungarotoxin. The binding is saturable and is inhibited by a variety of cholinergic agonists and antagonists. The pseudo-first order rate constant for binding is $2.1 \times 10^7$ M$^{-1}$ s$^{-1}$ at 22°C. In contrast to the α-bungarotoxin binding reaction found with muscle, the binding to PC12 is reversible with a first order rate constant of $4.9 \times 10^5$ s$^{-1}$ at 37°C. Toxin binds to an integral membrane component which sediments in sucrose gradients containing Triton X-100 with an apparent sedimentation coefficient of 10.5 S. The nicotinic acetylcholine receptor of PC12 was assayed by determining the agonist-induced increase in permeability to sodium ions. Using this assay, we determined the apparent binding constants for a variety of cholinergic ligands and found no correlation between their ability to affect cholinergic function and to inhibit binding of α-bungarotoxin. Therefore, the site at which cholinergic ligands affect receptor function is different than the site at which cholinergic ligands inhibit toxin binding.

Elapid neurotoxins bind specifically and with high affinity to acetylcholine receptors on muscle cells (1–5) and the electroplax of various electric fishes (6, 7). Neurotoxin bound to these receptors inhibits agonist-induced activation (1–8). The acetylcholine receptors responsible for cholinergic transmission in sympathetic ganglia and in the central nervous system differ from muscle acetylcholine receptor in their response to a variety of ligands. In particular, the agonist-induced activation of these receptors is insensitive to elapid neurotoxins (8, 9). One such toxin, α-bungarotoxin, does bind to sympathetic ganglion and membrane fragments prepared from brain at a site which is protected by cholinergic ligands (11–15). It has not however been demonstrated that the membrane component on these tissues which binds αBT$^\mathrm{I}$ is a cholinergic receptor. In fact, binding of αBT might result from several different situations. (a) There may be a variety of cell surface components which recognize and bind αBT. (b) The cells may synthesize a unique toxin-binding macromolecule distinct from the acetylcholine receptor. (c) The ganglionic and central nervous system acetylcholine receptors may bind neurotoxin in a manner which does not inhibit agonist-induced receptor activation. In this paper, we describe the binding of αBT to a rat sympathetic nerve cell line (16) and provide evidence that helps to distinguish between these alternatives.

**MATERIALS AND METHODS**

**Growth of Cells** – The rat sympathetic nerve cell line, PC12, was obtained from Greeno and Tischler (16) and was grown on plastic tissue culture dishes in Dulbecco modified Eagle's medium containing 10% fetal calf serum and 10% horse serum at 37°C in 12% carbon dioxide and 88% air. Under these conditions, the cells had a generation time of about 30 h. Cultures were transferred by aspirating the growth medium, adding new growth medium and removing the cells from the dish by pipetting. They were then replated at an density of 104/cm$^2$ on 100-mm dishes for generation of large quantities of cells or on 35-mm dishes for binding assays. Since the cells do not bind tightly to the dish and tend to grow in clumps we also used polylysine coated tissue culture dishes (17) in a number of instances. BC3H-1, a nonfusing muscle cell line (18), was handled as described previously (19).

**Preparation and Iodination of α-Bungarotoxin** – α-Bungarotoxin was purified from the venom of Bungarus multicinctus (Ross Allen Serpentarium) and iodinated using the iodine monochloride method described by Vogel et al. (20). Mono- and diiodo toxins were separated on CM-Sephadex (Pharmacia) and the diiodo derivative was used throughout these experiments. The specific activity of the αBT obtained had specific activities between 1 and $2 \times 10^6$ cpm/mmol.

**Assay for Toxin Binding to Cells** – The toxin binding capacity of cells was determined using the same procedures described previously for other cell lines (2, 19). Cells were removed from the incubator and placed in Prep medium containing 1% fetal calf serum. Cultures used to determine nonspecific binding were preincubated for 10 min with either 400 µM d-tubocurarine or 200 µM nicotine. 125I-αBT was then added and the cells were incubated at room temperature. The reaction was stopped by washing twice with Prep medium containing 1% fetal calf serum. Cells were removed from the dish by scraping with a plastic policeman, collected on Celox filters (Millipore), and washed with 8 ml of NaCl/P. The filters were placed into a scintillation fluid containing 50 ml of BBS-3 (Beckman) and 4 g of diphenoxazole per liter of toluene and were counted in a liquid scintillation counter.

**Sodium Flux Assay** – Agonist-induced sodium uptake was measured as described by Catterall (21) and by Stallcup and Cohn (22). The assay buffer contained 150 mM NaCl, 5.4 mM KCl, 2 mM CaCl$_2$, 5 mM glucose, and 50 mM Hepes adjusted to pH 7.4. Ouabain...
was included at a concentration of 5 mM and $^{3}H$-BT was added to a final level of 1 to 2 $\mu$Ci/ml. Sodium uptake was initiated by the addition of the desired agonist.

When the effects of antagonists were to be tested, the cells were incubated with the desired concentration of antagonist prior to addition of agonist. In the case of d-tubocurarine, atropine, and hexamethonium, this preincubation was performed at 22° for 10 min. For quinclidinyl benzilate the pre-incubation was allowed to proceed for 30 min. At the end of this time, the incubation medium was removed and replaced with assay medium which also contained the antagonist. In these cases the agonist was used at sub saturating levels so that possible inhibition would be maximal.

RESULTS

Characterization of Toxin Binding Reaction — We first characterized the reaction between aBT and PC12 by considering only that binding which was inhibited by d-tubocurarine. Cells were incubated with various concentrations of $^{3}H$-aBT at room temperature either in the presence or absence of 400 $\mu$M d-tubocurarine. After 1 h, the cells were washed free of unbound toxin, harvested on filters, and the amount of $^{3}H$-aBT bound was determined. The results in Fig. 1 show that $^{3}H$-aBT binds to these cells in the presence of 400 $\mu$M d-tubocurarine such that the binding increases linearly over the range of $^{3}H$-aBT concentrations employed. In contrast, the d-tubocurarine protectable binding is saturable, with maximum binding being achieved with a 60-min incubation in the presence of 0.01 $\mu$M $^{3}H$-aBT. At saturation, the cells have bound an average of $5 \times 10^{5}$ toxin molecules per cell or about 5 per square micron.

The rate of binding of $^{3}H$-aBT was found to follow pseudofirst order kinetics. The data in Fig. 2 show the amount of d-tubocurarine protectable $^{3}H$-aBT bound to PC12 cells as a function of time in the presence of 0.01 $\mu$M toxin. The pseudofirst order rate constant calculated from this data is 2.1 $\times 10^{3}$ M$^{-1}$ s$^{-1}$ which is similar to that found for binding of $^{125}$I-aBT to skeletal muscle (2) and to membrane fragments prepared from eel electric organ (23) or chick sympathetic ganglion (15). The reaction is first order over almost 3 orders of magnitude, suggesting that the binding occurs to a single class of sites.

The binding of $^{125}$I-aBT to muscle nicotinic acetylcholine receptors is not readily reversible, while Groene (15) has shown that complexes of $^{125}$I-aBT and sympathetic ganglion membrane fragments dissociate. We measured the reversibility of binding of toxin to PC12 cells and determined that the loss of toxin from the cells was not due to degradation of toxin-receptor complexes. A number of cultures were labeled to saturation with $^{125}$I-aBT and then returned to the incubator in growth medium. Several sets of cultures were supplemented with either cycloheximide (50 $\mu$g/ml), carbonyl-cyanide chlorophenylhydrazone (10 $\mu$M), or unlabeled aBT (0.1 $\mu$M). Cultures were removed as a function of time and the amount of $^{125}$I-aBT remaining bound to the cells was determined. The results in Fig. 3 show that the amount of bound toxin decreased in a first order process and was insensitive to any of the additions made. The first order rate constant for dissociation is 4.9 $\times 10^{-3}$ s$^{-1}$. The fact that the loss of radioactivity from the cells was independent of energy makes it unlikely that the loss occurred through degradation of $^{125}$I-aBT receptor complexes, since this process is known to be energy-dependent (24). In addition, degradation of $^{125}$I-aBT results in the release, into the medium, of $^{125}$I-tyrosine. We examined the material released from PC12 by chromatography on Bio-Gel P2 columns as previously described (19) and found that this degradation product accounted for less than 15% of the released isotope.

Properties of the Toxin Binding Component—The molecule which binds $^{125}$I-aBT is not released from the particulate fraction of cell homogenates by washing in 1 M NaCl. It is, however, found in the supernatant after extracting salt-washed membrane fragments with Triton X-100 (data not shown). The detergent-solubilized toxin binding component of PC12 migrates in sucrose gradients containing Triton X-100 (0.5%) as a single peak. Cells were labeled with $^{125}$I-aBT in

![Fig. 1. Concentration dependence of binding of $^{125}$I-aBT to cultures of PC12. A number of cultures on 35-mm tissue culture dishes were placed in 1 ml of Prep medium containing 1% fetal calf serum. One-half of the cultures then received d-tubocurarine to a final concentration of 400 $\mu$M and incubation was continued for 10 min. $^{125}$I-aBT was then added to cultures to give the indicated concentrations. At each concentration, two dishes contained only $^{125}$I-aBT and two dishes contained $^{125}$I-aBT plus 400 $\mu$M d-tubocurarine. Incubation was then continued for 1 h at room temperature, at which time the cells were washed twice with Prep medium containing 1% fetal calf serum, removed from the dish with a plastic polystyrene, collected on celoitate filters, and counted. $\bullet$—$\bullet$, amount of $^{125}$I-aBT bound in the presence of 400 $\mu$M d-tubocurarine; $\bigcirc$—$\bigcirc$, amount of $^{125}$I-aBT bound minus the amount bound in the presence of d-tubocurarine.

![Fig. 2. Time course of binding of aBT to PC12. Cells on 35-mm dishes were placed in Prep medium containing 1% fetal calf serum. One-half of the cultures were incubated for 10 min with 400 $\mu$M d-tubocurarine and then all the cultures received $^{125}$I-aBT at 0.01 $\mu$M. At the indicated times duplicate cultures, two with toxin and two with toxin plus d-tubocurarine, were washed and the cells were collected in celoiate filters. The curve shows the amount of d-tubocurarine protectable binding that had occurred after each time interval. The inset shows the same data plotted as the log of the unoccupied binding sites remaining as a function of time; the line was drawn by eye.]
the presence or absence of 400 \( \mu M \) \( d \)-tubocurarine, harvested, and extracted with NaCl/P, containing 3% Triton X-100. Both extracts were then sedimented in 5 to 20% sucrose gradients. At the same time, we ran parallel gradients containing proteins of known sedimentation coefficients. The data in Fig. 4 show that the \( d \)-tubocurarine protectable toxin binding component migrates in a single symmetrical peak with a sedimentation coefficient of 10.5 S. The nonspecific binding component migrates ahead of free toxin and thus does not represent toxin that is incompletely removed in the cell washing procedure. These results, in conjunction with the binding data, indicate that toxin binds to a single membrane component.

**Pharmacological Characterization of the Toxin Binding Component**—If \( \alpha \)-BT binds to an acetylcholine receptor on PC12, then the binding reaction should be inhibited by cholinergic ligands. Furthermore, the apparent dissociation constant for a ligand determined by its ability to inhibit toxin binding should be the same as that determined for that ligand by its ability to affect receptor function. As a first step in making this comparison we tested the ability of several cholinergic ligands to decrease the initial rate of toxin binding and receptor function requires an assay for receptor activation. Agonist-induced activation of acetylcholine receptors on excitable cells in tissue culture can be conveniently assayed by measurement of changes in membrane permeability to radioactive ions (21, 22). We have used this technique to assay activation of the nicotinic acetylcholine receptor in PC12, and to determine dissociation constants for several cholinergic ligands.

The results in Fig. 5 show the concentration dependence of carbamylcholine-induced activation and yield an apparent dissociation constant for this ligand of 700 \( \mu M \) at 22\(^\circ\). Similar experiments using nicotine as an agonist revealed an apparent

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**Table I**

| Ligand                  | PC12  | Rat brain* | Sympathetic ganglion* | BCSH-1C | Estradiol
|-------------------------|-------|------------|-----------------------|---------|-----------
| Carbamylcholine         | 200   | 100        | 100                   | 30      | 20        |
| Dicethoximine           | >400  | 900        | 900                   | 50      | 0         |
| Hexamethonium           | >400  | >1000      | 500                   | 80      | 60        |
| Nicotine                | 2     | 2          | 6                     | 60      | 20        |
| Benzoquinonium          | 7     | 2          |                        | 2       | 0.8       |
| \( d \)-Tubocurarine    | 3     | 1          | 2                     | 0.5     | 0.2       |
| Atropine                | >800  | 1000       | >1000                 | 200     |           |

* Data from Ref. 10.
* Data from Ref. 14.
* Data from Ref. 19.
* Data from Ref. 23.
Elapid neurotoxins have been shown to bind to nicotinic acetylcholine receptor from muscle and electric organs of eels and Torpedo. The evidence that the binding occurs at acetylcholine receptor is based on the following observations. (a) Binding is inhibited by cholinergic ligands and their ability to inhibit binding parallels their ability to activate or inhibit receptor function. (b) Binding of αBT to these receptors inhibits agonist-induced activation. (c) Antibodies prepared against a membrane component purified on the basis of its ability to bind toxin inhibit agonist-induced receptor activation.

A number of laboratories have documented binding of αBT to sympathetic ganglion membranes and to membrane fragments derived from brain. Although the ability of several

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\text{TABLE II}
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| Ligand              | Apparent dissociation constant \( \mu \text{M} \) | Ratio  |
|---------------------|--------------------------------------------------|--------|
| Carbamylcholine     | 200                                              | 3.3    |
| Nicotine            | 20                                               | 40     |
| d-Tubocurarine      | 80                                               | 30     |
| Hexamethonium       | >400                                             | 0.33   |
| Quinuclidinyl benzilate | >400                                          | <0.15  |
| Atropine            | >800                                             | 0.005  |

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\text{FIG. 5 (left). Concentration dependence of carbamylcholine-induced activation of acetylcholine receptors on PC12. Cultures of PC12 on 35-mm tissue culture dishes were placed in assay buffer and carbamylcholine was added at the indicated concentrations. The initial rate of uptake of \(^{22}\text{Na}\) was determined and plotted as a function of the concentration of carbamylcholine. The apparent dissociation constant for carbamylcholine determined from this experiment is } 7 \times 10^{-4} \text{ M.}
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\text{FIG. 6 (right). Inhibition of carbamylcholine-induced activation by cholinergic antagonists. Cultures of PC12 on 35-mm tissue culture dishes were placed in assay buffer and a cholinergic antagonist was added to the desired concentration. In the case of d-tubocurarine and hexamethonium the cultures were incubated with antagonist for 10 min at 22\degree \text{C. For quinuclidinyl benzilate, the incubation was carried out for 30 min at 22\degree \text{C. At the end of the time, carbamylcholine was added to 400 } \mu \text{M and the initial rate of uptake of } \(^{22}\text{Na}\) \text{ was determined. The figure shows the per cent inhibition of carbamylcholine-induced receptor activation as a function of the concentration of antagonist. The apparent dissociation constants determined for these three ligands are tabulated in Table II along with dissociation constants determined for other antagonists determined under the same assay conditions. } \circ \circ \circ, \text{ d-tubocurarine; } \circ \circ \circ, \text{ quinuclidinyl benzilate; } \circ \circ \circ, \text{ hexamethonium.}
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\text{DISCUSSION}
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dissociation constant of 80 \( \mu \text{M} \). The ability of antagonists to inhibit carbamylcholine induced sodium flux was determined in the presence of 400 \( \mu \text{M} \) carbamylcholine. The results in Fig. 6 show the concentration dependence of the binding achieved by hexamethonium, d-tubocurarine, and quinuclidinyl enzilate. The apparent dissociation constants determined for a variety of ligands are summarized in Table II and compared to their dissociation constants determined by inhibition of the initial rate of toxin binding. Nicotine is 40-fold more effective in the toxin binding assay and quinuclidinyl benzilate is 200-fold more effective in the sodium flux assay. These results indicate that the site at which these ligands bind to affect receptor function is different than the site at which they bind to inhibit toxin binding. The simplest interpretation of these findings is that toxin does not bind to the membrane component responsible for ligand induced changes in permeselectivity.

Previously we demonstrated that concentrations of αBT sufficient to saturate the toxin binding sites of PC12 had no effect on acetylcholine receptor function (20), a property which PC12 shares with brain and sympathetic ganglia. This fact alone did not rule out the possibility that αBT might bind to this type of receptor without impairing its function. To rule out this alternative we made use of the fact that d-tubocurarine and αBT compete for a binding site on PC12 (see Table I). If this site is in fact on the acetylcholine receptor then αBT should interfere with the ability of d-tubocurarine to inhibit receptor function, even though αBT does not affect receptor function itself. We therefore redetermined the concentration dependence of d-tubocurarine inhibition of receptor function using cells that were saturated with αBT. The results in Fig. 7 show that saturation of the αBT binding sites with αBT has no effect on the ability of d-tubocurarine to inhibit receptor function. Again, this suggests that the site at which d-tubocurarine binds to inhibit toxin binding is different from the site at which d-tubocurarine binds to inhibit function.
cholinergic ligands to inhibit this binding was determined, the ability of these same ligands to effect receptor function remained unknown. This omission, in concert with the fact that αBT has no effect on agonist-induced activation of acetylcholine receptors in these tissues left the identity of the toxin binding component unknown. In this report we have tried to distinguish between several alternatives using a clonal sympathetic nerve cell line PC12.

We first established that the toxin binding component on PC12 had the same binding kinetics as that found on sympathetic ganglia and brain membrane fragments. In fact, these three preparations share several characteristics: (a) αBT binding to each is saturable, (b) they have the same rate constant for αBT binding, (c) they have similar rate constants for αBT dissociation, and (d) they have the same apparent dissociation constants for all the cholinergic ligands which we tested. These results make it seem very likely that the membrane component which binds toxin on PC12 is analogous to that which binds toxin on sympathetic ganglia and on brain fragments. Thus, conclusions drawn on the basis of experiments with PC12 should prove applicable to these other preparations.

The toxin binding component is probably an integral membrane protein since it is not removed from membrane fragments with salt but is extracted with nonionic detergent. It is probably a single molecular species since (a) the αBT binding reaction follows pseudo-first order kinetics over almost 2 orders of magnitude and (b) the αBT complex migrates as a single symmetrical peak in sucrose gradients. The sedimentation coefficient of the toxin binding component is 10.5 S, larger than that found for muscle nicotinic acetylcholine receptor (9,55).

Cholinergic ligands both inhibit toxin binding and affect acetylcholine receptor function. If both effects are a consequence of binding the cholinergic ligand at the same site on the membrane then the apparent dissociation constant determined for a given ligand in one assay should be the same as that determined in the other assay. When we compared a variety of ligands in both assays we found wide variations. For example, the affinity with which hexamethonium binds to inhibit carbamylcholine-induced receptor activation. The simplest interpretation of these results is that αBT binds to a membrane component which is different than the acetylcholine receptor.

We have been able to test this hypothesis in a second fashion, based on the fact that αBT is unable to affect receptor function but does compete with d-tubocurarine for a binding site in PC12. If the ability of d-tubocurarine to inhibit receptor function and to block toxin binding is the result of d-tubocurarine binding to a single site, then αBT should protect the receptor against d-tubocurarine inhibition. In fact no such protection is observed. Once again we take these results to mean that the d-tubocurarine binding site which inhibits receptor function is different than the one which blocks toxin binding.

Our results suggest that αBT binds to a membrane component on PC12, and by analogy on sympathetic ganglia and brain fragments, which is different than the functional nicotinic acetylcholine receptor. This conclusion is consistent with our studies which show that anti-acetylcholine receptor antibodies block nicotinic acetylcholine receptor activation on PC12 but fail to recognize the membrane component which binds αBT (26).

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