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 × 10^8 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 × 10^-5 s^-1 at 37°C. Toxin binds to an integral membrane component which sediments at 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 electrophysiology 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 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, 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 about 10^4/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. The cells were harvested and replated on polylysine coated dishes and used for binding or sodium flux experiments within 2 days. All the sodium flux assays were done with cells on polylysine coated dishes. 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 diido toxos were separated on CM-Sephadex (Pharmacia) and the diido derivative was used throughout these experiments. The 125I-αBT obtained had specific activities between 1 and 2 × 10^8 cpm/nmol.

**Assay for Toxin Binding to Cells**—The toxin binding capacity of cell cultures 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 pretreated with Prep medium containing 1% fetal calf serum. Cells were removed from the dish by scraping with a plastic policeman, collected on Celotex 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 130 mM NaCl, 5.4 mM KCl, 2 mM CaCl₂, 5 mM glucose, and 50 mM Hepes adjusted to pH 7.4.

**References**

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3. The abbreviations used are: αBT, α-bungarotoxin; I-αBT, diido-α-bungarotoxin; NaCl/P, 138 mM NaCl, 3 mM KCl, 0.9 mM CaCl₂, 0.05 mM MgCl₂, 8.15 mM NaH₂PO₄, Prep medium, Dulbecco modified Eagle's medium in which the bicarbonate is replaced with 1.08 mM Na₂HPO₄, 1.15 mM KH₂PO₄; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

4. The work was supported by grants from the National Institutes of Health, Muscular Dystrophy Associations, Inc., and National Foundation-March of Dimes, and National Science Foundation Grant BNS 77-01548. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

5. The abbreviations used are: αBT, α-bungarotoxin; I-αBT, diido-α-bungarotoxin; NaCl/P, 138 mM NaCl, 3 mM KCl, 0.9 mM CaCl₂, 0.05 mM MgCl₂, 8.15 mM NaH₂PO₄, 1.15 mM KH₂PO₄; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
The reaction between aBT and PC12 by considering only that binding which was inhibited by d-tubocurarine. Cells were incubated with various concentrations of 125I-aBT at room temperature either in the presence or absence of 400 μM d-tubocurarine. After 1 h, the cells were washed free of unbound toxin, harvested on filters, and the amount of 125I-aBT bound was determined. The results in Fig. 1 show that 125I-aBT binds to these cells in the presence of 400 μM d-tubocurarine such that the binding increases linearly over the range of 100-500 μM 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 μM 125I-aBT. At saturation, the cells have bound an average of 6.1 x 10^4 toxin molecules per cell or about 3 per square micron.

The rate of binding of 125I-aBT was found to follow pseudo-first order kinetics. The data in Fig. 2 show the amount of d-tubocurarine protectable 125I-aBT bound to PC12 cells as a function of time in the presence of 0.01 μM toxin. The pseudo-first order rate constant calculated from this data is 2.1 x 10^5 M^-1 s^-1 which is similar to that found for binding of 125I-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 2 orders of magnitude, suggesting that the binding occurs to a single class of sites.

Properties of the Toxin Binding Component - The molecule which binds 125I-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 125I-aBT in
the presence or absence of 400 \( \mu M \) \( d \)-tubocurarine, harvested, and extracted with NaCl/IP 

extracted with NaCl/IP 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 determined the concentration of ligand which reduces this initial rate by one-half, a concentration which approximates the apparent dissociation constant for that ligand (25).

The results of a number of such experiments are tabulated in Table I and compared with other tissues. 

The results which we obtained are similar to those obtained with sympathetic ganglia and with brain, and suggest that the toxin binding component in these three tissues is the same. 

Assay for Cholinergic Function in PC12—The second step in comparing the ability of cholinergic ligands to affect 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). 

For example, addition of carbamylcholine to the clonal muscle cell line L6 results in increased permeability to sodium ions (22). We have used this technique to assay activation of the nicotinic acetylcholine receptor present on 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°. Similar experiments using nicotine as an agonist revealed an apparent
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 from muscle and electric organs of eels 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 α-Bungarotoxin and Cholinergic Function

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**Table II**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Apparent dissociation constant in μM</th>
<th>Toxin binding K&lt;sub&gt;M&lt;/sub&gt;</th>
<th>Sodium flux assay K&lt;sub&gt;M&lt;/sub&gt;</th>
<th>Ratio</th>
</tr>
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<tbody>
<tr>
<td>Carbacholymoline</td>
<td>200</td>
<td>100</td>
<td>3.3</td>
<td>1.0</td>
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<tr>
<td>Nicotine</td>
<td>2</td>
<td>80</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>d-Tubocurarine</td>
<td>3</td>
<td>1</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Hexamethonium</td>
<td>&gt;400</td>
<td>60</td>
<td>&lt;0.15</td>
<td></td>
</tr>
<tr>
<td>Quinuclidinyl benzilate</td>
<td>&gt;400</td>
<td>2</td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>&gt;800</td>
<td>6</td>
<td>&lt;0.0076</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

The dissociation constant of 80 μM. The ability of antagonists to inhibit carbacholymoline induced sodium flux was determined in the presence of 400 μM carbacholymoline. The results in Fig. 6 show the concentration dependence of the inhibition 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 from 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 permselectivity.

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.

**Fig. 5** (left). Concentration dependence of carbacholymoline-induced activation of acetylcholine receptors on PC12. Cultures of PC12 on 35-mm tissue culture dishes were placed in assay buffer and carbacholymoline was added at the indicated concentrations. The initial rate of uptake of 22Na<sup>+</sup> was determined and plotted as a function of the concentration of carbacholymoline. The apparent dissociation constant for carbacholymoline determined from this experiment is 7 × 10<sup>-11</sup> M.

**Fig. 6** (right). Inhibition of carbacholymoline-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 °C. For quinuclidinyl benzilate, the incubation was carried out for 30 min at 22 °C. At the end of the time, carbacholymoline was added to 400 μM and the initial rate of uptake of 22Na<sup>+</sup> was determined. The figure shows the per cent inhibition of carbacholymoline-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. O—O, d-tubocurarine; O—O, quinuclidinyl benzilate; O—O, hexamethonium.
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 cloning 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 receptor function is different than 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 both 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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J Patrick and B Stallcup


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