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Inhibition of neuronal acetylcholine sensitivity by α-toxins from *Bungarus multicinctus* venom

(ciliary ganglion neurons/acetylcholine receptors/rhodamine-labeled α-bungarotoxin/cell culture)

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ABSTRACT ***Bungarus multicinctus*** **venom contains several α-toxins in addition to the widely used α-bungarotoxin (Bgt 2.2). We have found that two of the α-toxins (Bgt 3.1 and 3.3) inhibit neuronal acetylcholine (AcCho) sensitivity when tested on ciliary ganglion neurons in cell culture. Over 90% of the AcCho sensitivity recorded in response to iontophoretic application of AcCho was blocked when the neurons were incubated with either of the toxins at 10^{-7} M for 1 hr at 37°C. The blockade could be partially reversed by incubating the neurons for 1-2 hr in medium lacking the toxins. The neurons also had a high-affinity binding site for Bgt 2.2, as indicated by binding studies with rhodamine-labeled Bgt 2.2. Concentrations of Bgt 2.2 (10^{-7} M) that should be nearly adequate to saturate the high-affinity site, however, had no detectable effect on AcCho sensitivity of the neurons. Higher concentrations of Bgt 2.2 (10^{-5} M) produced a partial inhibition of AcCho sensitivity, suggesting either that the neurons had two classes of binding sites for Bgt 2.2 (with the low-affinity site affecting AcCho sensitivity) or that the preparation of Bgt 2.2 contained minor components (e.g., Bgt 3.1 or 3.3) that were responsible for the blockade. The mechanisms by which Bgt 3.1 and 3.3 inhibit neuronal AcCho sensitivity remain unknown. If they bind specifically to the AcCho receptor, they will be useful agents for studying the distribution and regulation of this membrane component.**

α-Bungarotoxin [Bgt 2.2 (1)], a small protein toxin isolated from the venom of the elapid snake *Bungarus multicinctus*, binds tightly and specifically to nicotinic acetylcholine (AcCho) receptors in vertebrate skeletal muscle. The specificity of Bgt 2.2 binding has been demonstrated by showing that Bgt 2.2 binding blocks AcCho sensitivity in skeletal muscle, that it codistributes with AcCho sensitivity along the surface of the muscle fiber, and that it is blocked by ligands that bind to the nicotinic receptor. These properties have made the toxin a valuable agent for studying the muscle AcCho receptor.

A number of laboratories have reported that Bgt 2.2 also binds specifically to vertebrate neuronal membranes which contain nicotinic AcCho receptors (see ref. 2 and refs. therein). The binding occurs with high affinity and is blocked by ligands that bind to the neuronal nicotinic receptor. The identity of the Bgt 2.2 binding component, however, remains unclear since concentrations of Bgt 2.2 adequate to saturate the binding site have no demonstrable effect on AcCho receptor function for the neurons. Examples include chick sympathetic neurons in dissociated cell culture or *in vivo* (2, 3), rat superior cervical ganglia (4), and the pheochromocytoma cell line PC12 (5). The strongest evidence suggesting that the Bgt 2.2 binding site may be different from the neuronal AcCho receptor comes from immunological studies on PC12 cells. Antibodies prepared against eel AcCho receptors did not crossreact with the Bgt 2.2 binding component in PC12 cells, but the antibodies did block AcCho receptor function in the cells and did crossreact with a component in the cell homogenate different from the component that bound Bgt 2.2 (6). It has been reported that high concentrations of Bgt 2.2 (10^{-6} M) can reversibly block nicotinic transmission through the ciliary ganglion (7). An earlier study found no effect of Bgt 2.2 on transmission through the ganglion (8).

B. multicinctus venom contains several α-toxins that block AcCho receptors in skeletal muscle (1, 9) in addition to the widely used Bgt 2.2. We examined the effects of these toxins on ciliary ganglion neurons in cell culture to determine if they could also block neuronal AcCho receptors. Two of the α-toxins (Bgt 3.1 and 3.3) were very effective in blocking sensitivity of the neurons to iontophoretically applied AcCho. Studies with rhodamine-labeled Bgt 2.2 demonstrated that the neurons also had a high-affinity binding site for Bgt 2.2, but concentrations of Bgt 2.2 adequate to saturate the site did not inhibit AcCho sensitivity.

MATERIALS AND METHODS

Preparation of Cell Cultures. Ciliary ganglion neuron–myotube cultures were prepared and maintained as described (10). Briefly, ciliary ganglia from 8-day-old chicken embryos were dissociated and plated at a density of about 10^4 neurons on 5-day-old myotube cultures (35-mm dishes) prepared from embryonic chicken pectoral muscle. The cultures were grown for 1-2 weeks in culture medium containing Eagle's minimal essential medium supplemented with 10% (vol/vol) horse serum, 5% (vol/vol) chicken embryo extract, 50 units of penicillin per ml, and 50 μg of streptomycin per ml.

For experiments with fluorescence microscopy, glass-bottomed culture dishes were prepared by drilling an 18-mm hole in a 35-mm plastic culture dish and attaching a 25-mm Vanlab glass coverslip with Sylgard. The surface was coated with collagen and sterilized by UV irradiation. Cells were then plated and maintained in the cultures as described above.

Purification of α-Toxins. α-Toxins from *B. multicinctus* venom were purified by ion-exchange chromatography as described (1), with the following modifications. The venom (200 mg in 6 ml) was applied to a column (40 × 2.5 cm) of carboxymethyl-Sephadex C-50 equilibrated with 50 mM ammonium acetate (pH 5.0). The column was eluted at 22 ml/hr with a 500-ml linear gradient of 50 mM ammonium acetate (pH 5.0) to 1.0 M ammonium acetate (pH 6.9). Fractions for peaks II and III (1) were pooled separately, lyophilized, and redissolved in 0.1 M ammonium acetate (pH 6.9). Further fractionation was achieved by chromatography on Whatman CM32 carboxymethyl-cellulose. Peak II was applied to a column (30 × 1 cm) equilibrated with 0.1 M ammonium acetate (pH 6.9) and was eluted at 7 ml/hr with a 160-ml linear gradient of 0.1–0.3 M

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Abbreviations: Bgt 2.2, α-bungarotoxin; Bgt 2.1 and Bgt 3.1–3.4, other α-type toxins from *Bungarus multicinctus* venom; AcCho, acetylcholine; $NaDodSO_4$, sodium dodecyl sulfate.

ammonium acetate (pH 6.9). Peak III was fractionated similarly on a separate column. Material from individual peaks from the carboxymethyl-cellulose columns were pooled, lyophilized, redissolved in 2 mM sodium phosphate, pH 7.5/0.15 M NaCl, and stored frozen. Toxin concentrations were calculated by assuming a millimolar extinction coefficient of 5 at 280 nm.

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed in 15% polyacrylamide slab gels (9 × 13 × 0.1 cm) containing 0.1% $NaDodSO_4$ (11). Samples were prepared by boiling them for 3 min in 60 mM Tris, pH 6.8/5% mercaptoethanol/3% $NaDodSO_4$/10% glycerol. Mercaptoacetic acid (0.01%) was added to the upper reservoir buffer. Electrophoresis was carried out at 20 mA for 5 hr. Bromphenol blue was used as the tracking dye. Gels were stained with Coomassie blue. Molecular weight standards included chymotrypsin, carbonic anhydrase, hemoglobin, lysozyme, Bgt 2.2, and insulin.

Fluorescence Microscopy. Cultures treated with tetramethyl rhodamine-conjugated Bgt 2.2 were examined with a Leitz Diavert microscope equipped with a Ploemopak attachment and Leitz Filter System N for viewing rhodamine fluorescence. A mercury lamp was used for illumination; a 1.3 numerical aperture 40 × oil immersion objective was used for viewing the cells.

Electrophysiology. Electrophysiological experiments were performed on the stage of a Leitz inverted microscope equipped with phase-contrast optics. Cultures were maintained at 35–36°C in Eagle's minimal medium supplemented with 1 mM $CaCl_2$, 10 mM sodium acetate, 22 mM glucose, 50 units of penicillin per ml, and 50 μg of streptomycin per ml. The pH was maintained at 7.2–7.4 by passing CO_2 over the culture. Microelectrodes (70–120 MΩ) filled with 3 M potassium acetate were used for intracellular recording from neurons and myotubes. AcCho sensitivities were determined by using micropipets (60–120 MΩ) filled with 3 M AcCho to iontophoretically apply AcCho to the surface of cells. Small, positive current pulses (5–50 nA, 2–10 msec) were used to deliver the AcCho while a steady negative current (2–4 nA) was maintained to prevent leakage of AcCho between pulses. The tip of the AcCho micropipette was positioned by lowering it until movement in a horizontal plane allowed it to touch the apparent periphery of the cell and then adjusting its position in the horizontal plane for maximum response. Pulses of different amplitudes but constant duration were applied to each cell to generate a "dose–response" curve for determining sensitivity to AcCho. Responses greater that 5 mV were excluded in computation of sensitivities. Cells with resting potentials less than 40 mV were rejected. The input resistance of cells was estimated by using a single microelectrode. Mean values of 49 ± 2 mV (SEM, n = 35) and 42 ± 8 MΩ (SEM, n = 34) were obtained for the resting potential and input resistance, respectively, for the ciliary ganglion neurons in culture. Action potentials were triggered in the neurons by intracellular stimulation as described (10).

Chemicals and Media. *B. multicinctus* venom was purchased from Miami Serpentarium and from Sigma. Tetramethyl rhodamine-conjugated Bgt 2.2 was prepared as described (12) by using tetramethyl rhodamine isothiocyanate purchased from Research Organics, Inc. (Cleveland, OH). *d*-Tubocurarine, AcCho, and cytosine arabinoside were purchased from Sigma; carboxymethyl-Sephadex C-50 from Pharmacia; CM32 carboxymethyl-cellulose from Whatman; and acrylamide, *N,N'*-methylene bisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine (Temed) from BioRad. Culture media and biological materials were obtained and prepared as described (10).

RESULTS

α-Toxins from *B. multicinctus* Venom. α-Toxins were purified from venom by ion-exchange chromatography. The venom was first chromatographed on carboxymethyl-Sephadex as described (1) (Fig. 1*A*). Peaks II and III were chosen for further fractionation since previous studies showed that they contained α-toxins, as indicated by their ability to block the AcCho sensitivity of skeletal muscle (1). When fractionated by chromatography on carboxymethyl-cellulose, peak II was resolved into two major components designated Bgt 2.1 and Bgt 2.2 (Fig. 1*B*). Bgt 2.2 corresponds to component II_2 described by Lee *et al.* (1) and is the α-toxin widely used as "α-bungarotoxin." Peak III from the initial fractionation was also chromatographed on carboxymethyl-cellulose and was resolved into four distinct components (Fig. 1*C*). These have been designated Bgt 3.1, 3.2, 3.3, and 3.4 in the order of their elution. Bgt 3.3 and 3.4 were the major constituents of peak III. Bgt 3.1 and 3.2 were present in variable amounts in different batches of venom and were always minor components.

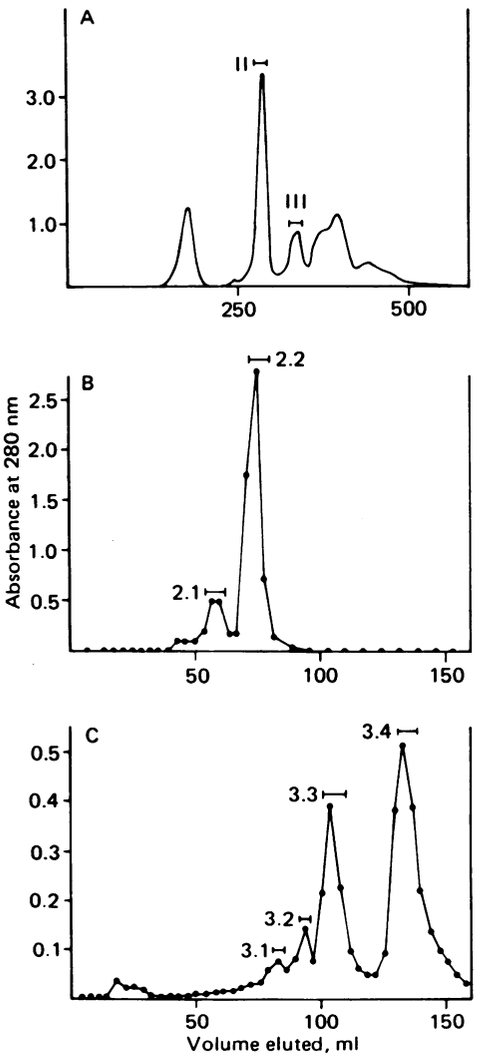

FIG. 1. Fractionation of *B. multicinctus* venom by ion-exchange chromatography. (*A*) Column chromatography of unfractionated venom on carboxymethyl-Sephadex C-50. Horizontal bars, fractions pooled for peaks II and III. (*B*) Column chromatography of peak II on carboxymethyl-cellulose. Horizontal bars, pooled fractions for components Bgt 2.1 and 2.2. (*C*) Column chromatography of peak III on carboxymethyl-cellulose. Horizontal bars, pooled fractions for components Bgt 3.1, 3.2, 3.3, and 3.4.

The purified α-toxins were examined by $NaDodSO_4$/polyacrylamide gel electrophoresis to estimate their homogeneity and to compare their polypeptide molecular weights. In each case a single major band of material was present on the gel, suggesting that the preparation was at least 90% homogeneous with respect to size (Fig. 2). The components fell into three classes on the basis of molecular weights. Bgt 2.1 was the largest, with an apparent molecular weight of greater than 10,000. Bgt 2.2, 3.2, and 3.3 had molecular weights of about 8000. Bgt 3.1 and 3.4 were the smallest and had molecular weights of about 6500.

Binding of Rhodamine-Labeled Bgt 2.2. Ciliary ganglion neurons in cell culture have a high-affinity binding site for Bgt 2.2. This was shown by incubating the neurons with 10^{-7} M tetramethyl rhodamine-conjugated Bgt 2.2 for 1 hr at 37°C, rinsing the neurons, and then examining them by fluorescence microscopy (Fig. 3). Many neurons were brightly labeled over the cell body and neurites, while other neurons in the same cultures were only dimly labeled. This apparent heterogeneity in site densities for Bgt 2.2 binding sites among neurons persisted with culture age. The binding of fluorescent Bgt 2.2 was almost completely blocked when cultures were first preincubated for 1 hr in 10^{-8} M unlabeled Bgt 2.2 and then were incubated for 1 hr in 10^{-7} M rhodamine-Bgt 2.2 plus 10^{-8} M unlabeled Bgt 2.2. These results are consistent with a dissociation constant of less than 10^{-8} M for Bgt 2.2 binding, suggesting that 10^{-7} M Bgt 2.2 binding should nearly saturate the site. Rhodamine-Bgt 2.2 binding was also largely blocked when 10 μM *d*-tubocurarine or 10 μM nicotine was included in the incubation medium; 10 μM atropine had no detectable effect.

AcCho Sensitivity of Ciliary Ganglion Neurons. The effects of the purified α-toxins on neuronal AcCho receptors were examined by using iontophoretically applied AcCho to measure AcCho sensitivities for ciliary ganglion neurons in the presence and absence of the toxin. The mean value of AcCho sensitivities for ciliary ganglion neurons in the absence of toxin was 75 ± 25 mV/nC (SEM, n = 35). As with rhodamine-Bgt 2.2 binding, there was considerable heterogeneity in AcCho sensitivities among neurons. The reason for the heterogeneity is not understood. An example of an AcCho response for a ciliary ganglion neuron is shown in Fig. 4*A*. The responses were almost completely blocked by 100 μM *d*-tubocurarine (Fig. 4*B*) or by 20 μM nicotine.

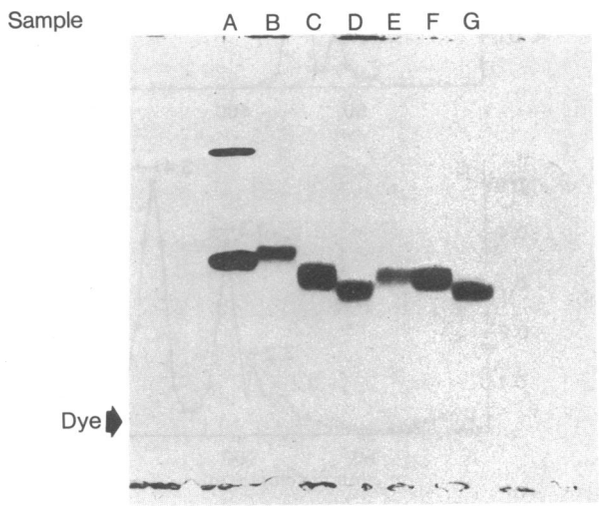

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified α-toxins. Samples of purified α-toxins (≈10 μg each) were prepared and subjected to $NaDodSO_4$/polyacrylamide slab gel electrophoresis. Electrophoresis was from top to bottom. Arrow indicates the position of the tracking dye at the end of the run. Lane A, standards: carbonic anhydrase and lysozyme; lane B, Bgt 2.1; lane C, Bgt 2.2; lane D, Bgt 3.1; lane E, Bgt 3.2; lane F, Bgt 3.3; lane G, Bgt 3.4.

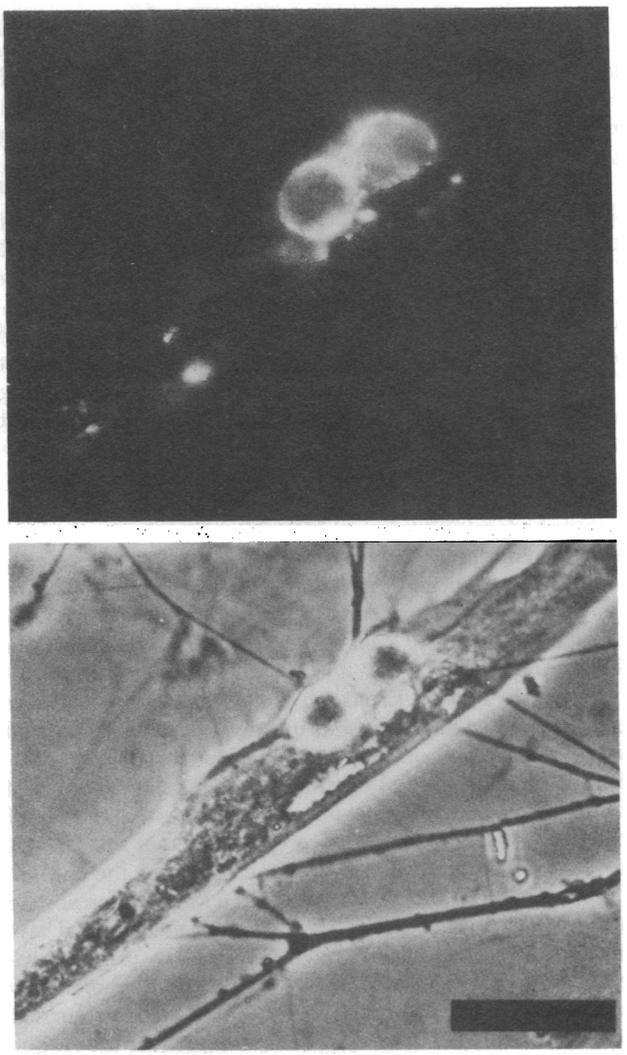

FIG. 3. Binding of rhodamine-labeled Bgt 2.2 to ciliary ganglion neurons in cell culture. A ciliary ganglion neuron–myotube culture was incubated with 10^{-7} M tetramethyl rhodamine-conjugated Bgt 2.2 in culture medium for 1 hr at 37°C, rinsed three times with medium, and viewed by fluorescence microscopy. (*Upper*) Fluorescence micrograph; (*Lower*) phase-contrast micrograph of same field of view. The fluorescence photograph was taken in a focal plane that emphasized labeling of the neuron cell bodies but was not favorable for viewing labeled neurites. Several high-density regions of toxin binding were also present on the myotube. Calibration bar, 50 μm.

The most potent inhibitors of AcCho sensitivity were Bgt 3.1 and 3.3. At 10^{-7} M, each blocked at least 90% of the AcCho sensitivity (Table 1). The toxins had no effect on the mean resting potential or input resistance, and they did not prevent spontaneous or evoked action potentials in the neurons. For both toxins, the blockade of sensitivity could be partially reversed by incubation of the cultures for 1–2 hr in medium lacking the toxin (Table 1). These results are consistent with the toxins' binding to the AcCho receptor with a dissociation constant of less than 10^{-7} M.

Partial inhibition of AcCho sensitivity was achieved by high concentrations of Bgt 2.2 (10^{-5} M), Bgt 3.2 (10^{-6} M), and Bgt 3.4 (10^{-6} M). Possibly Bgt 2.2, 3.2, and 3.4 act on AcCho re-

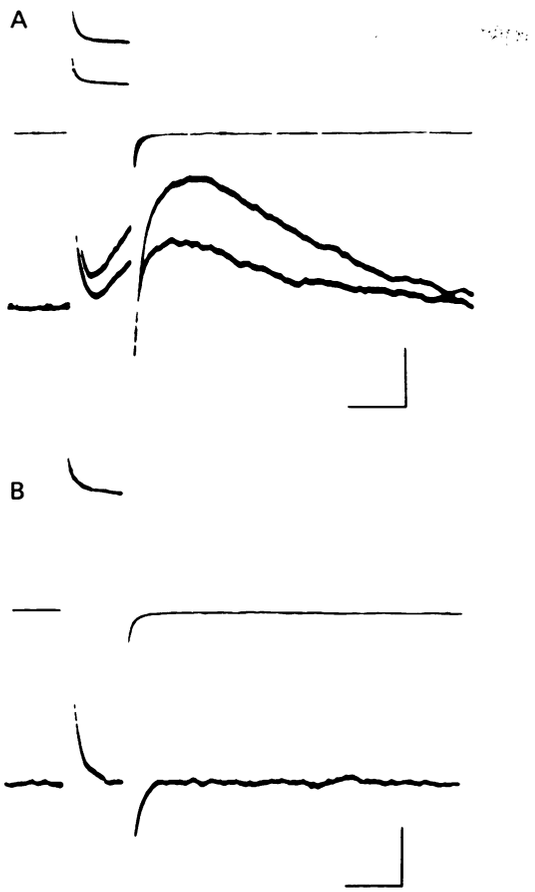

FIG. 4. AcCho sensitivity of a ciliary ganglion neuron in culture. AcCho sensitivity was examined by iontophoretically applying AcCho to the neuron while recording from it intracellularly. (*Upper*) Two superimposed trials in normal medium are shown. The upper traces represent the current being passed through the AcCho micropipet, while the lower traces indicate the response of the neuron. The larger current pulse corresponds to the larger response. (*Lower*) A single trial in medium containing 0.1 mM *d*-tubocurarine is shown for the same neuron. The upper trace indicates the current passed through the AcCho micropipet, while the lower trace shows the lack of a response in the neuron. Calibration bars: vertical, 20 nA or 5 mV; horizontal, 5 msec.

Table 1. Inhibition of neuronal AcCho sensitivity by α-toxins

Toxin	Remaining AcCho sensitivity, %
Bgt 2.1: 10^{-6} M	113 ± 40 (8)
Bgt 2.2: 10^{-5} M	39 ± 12 (43)*
10^{-6} M	40 ± 15 (13)*
10^{-7} M	88 ± 16 (24)
Bgt 3.1: 10^{-6} M	0 ± 1 (8)**
10^{-7} M	1 ± 1 (10)**
10^{-8} M	10 ± 4 (5)*
10^{-7} M and rinsed 1–2 hr	69 ± 17 (11)
Bgt 3.2: 10^{-6} M	5 ± 2 (8)**
10^{-7} M	33 ± 18 (16)**
Bgt 3.3: 10^{-6} M	1 ± 1 (36)**
10^{-7} M	9 ± 3 (17)**
10^{-6} M and rinsed 1–2 hr	51 ± 12 (10)*
Bgt 3.4: 10^{-6} M	17 ± 7 (14)**
10^{-7} M	60 ± 17 (17)

Ciliary ganglion–myotube cultures were incubated in medium containing the indicated concentration of α-toxin for 1 hr at 37°C. Neurons were then selected at random and tested for sensitivity to iontophoretically applied AcCho in the continued presence of the toxin. Values for individual cultures were averaged and expressed as a percent of the average value found in control cultures from the same cell plating examined on the same day. In some experiments, AcCho sensitivities were compared for neurons in the same culture before and after the incubation with toxin. Results obtained from different cell platings were then pooled and are expressed as a mean percent ± SEM. Numbers in parentheses indicate the number of neurons tested for each toxin concentration; neurons from two to eight cultures were used for each toxin concentration. When reversibility was to be tested (e.g., 10^{-7} M and rinsed 1–2 hr), AcCho sensitivities for neurons in the same culture were determined before incubation with toxin, in the presence of toxin after a 1-hr incubation, and after two to three rinses and 1–2 hr of incubation in medium lacking the toxin. Though SEM values are shown for all determinations, their interpretation is complicated by the fact that the distribution of AcCho sensitivities in cultures was unimodal but not normal. Accordingly, a nonparametric rank-sum test was applied to determine the confidence levels at which the determined values were different from controls; * denotes $P < 0.05$. and ** denotes $P < 0.001$.

ceptor function via a low affinity mechanism. Alternatively, the observed inhibition may reflect the presence of trace amounts of Bgt 3.1 or 3.3 in the toxin preparations. For example, the amount of inhibition observed with Bgt 2.2 could be accounted for by a 0.1% contamination with Bgt 3.1. In any case, it is clear that concentrations of Bgt 2.2 that should be adequate to saturate the high-affinity binding site revealed by rhodamine-Bgt 2.2 have no detectable effect on AcCho sensitivity. All of the Bgt α-toxin preparations greatly reduced or completely blocked AcCho sensitivities on skeletal myotubes in the ciliary ganglion neuron–myotube cultures when present at 10^{-6} M (data not shown).

DISCUSSION

The major finding presented here is that two α-toxins from *B. multicinctus* venom (Bgt 3.1 and 3.3) can block neuronal AcCho sensitivity. The blockade occurs at low concentrations of the toxins and is at least partially reversible. A specific action of the toxins on the AcCho receptor is suggested by the fact that other properties of the neurons, such as the resting potential, input resistance, electrical excitability, and general morphology, are not altered by the toxins. The mode of action of the toxins in blocking AcCho sensitivity, however, remains unknown, and additional studies are required to determine if they bind directly to the AcCho receptor.

Using rhodamine-labeled Bgt 2.2, we have found that ciliary ganglion neurons grown in dissociated cell culture have a high-affinity binding site for Bgt 2.2. Preincubating the neurons with 10^{-8} M unlabeled Bgt 2.2 blocked most of the rhodamine-labeled Bgt 2.2 binding, suggesting that the dissociation constant for the site was lower than 10^{-8} M. The rhodamine-Bgt 2.2 binding was also inhibited by low concentrations of nicotine and *d*-tubocurarine. Previous studies have shown that membrane preparations from ciliary ganglia have a high-affinity binding site for ^{125}I-labeled Bgt 2.2 (13, 14). The binding to membranes was saturable and could be blocked by ligands for the nicotinic receptor. We find, however, that a concentration of Bgt 2.2 that should be nearly adequate to saturate the neuronal sites observed in cell culture had no detectable effect on AcCho sensitivity for the neurons. Similar results were found for chicken sympathetic neurons in cell culture (2, 3) and for a pheochromocytoma cell line PC12 (5). In both cases the cells had a high-affinity binding site for Bgt 2.2 but the toxin did not

inhibit receptor function when bound to the site. These results, together with the immunological studies cited previously (6), raise questions about the relationship between the high-affinity binding site for Bgt 2.2 and the nicotinic AcCho receptor on neurons. Two electron microscopic studies have shown that horseradish peroxidase-conjugated Bgt 2.2 is retained at synaptic regions at a small fraction of the synapses in chicken retina (15) and rat brain (16). Thus, the Bgt 2.2 high-affinity binding component on neurons may have a synaptic function whether or not it is the AcCho receptor.

In contrast to the results with low concentrations of Bgt 2.2, we find that high concentrations do produce partial inhibition of AcCho sensitivity for ciliary ganglion neurons in culture. Chiappinelli and Zigmond (7) found that 10^{-6} M Bgt 2.2 reversibly blocked transmission through the ciliary ganglion in a manner consistent with blockade of the nicotinic receptors. Possibly, ciliary ganglion neurons have two binding sites for Bgt 2.2, with the low-affinity site being the nicotinic receptor. Alternatively, the Bgt 2.2 preparations may have been contaminated with trace amounts of Bgt 3.1 or 3.3 which could have produced the inhibition. The partial inhibition observed with Bgt 3.2 and 3.4 could also reflect either a low affinity of the toxins for the neuronal nicotinic receptor or a small contamination with Bgt 3.1 or 3.3.

Considerable heterogeneity was often observed both in the degree of AcCho sensitivity and in the amount of rhodamine-Bgt 2.2 binding among neurons in the same culture. No obvious morphological differences were apparent for neurons with different amounts of labeling or sensitivity, and the cells had similar resting potentials and input resistances. Heterogeneity for AcCho sensitivity has previously been observed for ganglionic neurons in cell culture (2, 17). We have not determined whether Bgt 2.2 binding and AcCho sensitivity codistribute among ciliary ganglion neurons.

All six of the toxins tested were referred to as α-type toxins because of previous work (1, 9) and because we found that the purified fractions blocked muscle AcCho sensitivity in culture. In some cases, such as Bgt 2.1, however, the classification remains tentative since it was less effective than others in blocking muscle sensitivity and might have contained trace amounts of Bgt 2.2 that could have accounted for the blockade.

It will be important to determine the mode of action for Bgt 3.1 and 3.3 in blocking neuronal AcCho sensitivity. If the toxins bind tightly and specifically to the neuronal nicotinic AcCho receptor, they should be useful agents for studying the distribution and regulation of the receptor during development and may also help elucidate the relationship between the receptor and the Bgt 2.2 binding site on neurons.

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