Ultrastructural Distribution of ^{125}I -Toxin F Binding Sites on Chick Ciliary Neurons: Synaptic Localization of a Toxin that Blocks Ganglionic Nicotinic Receptors

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Although α -bungarotoxin (BGT), a common probe for nicotinic ACh receptors on vertebrate skeletal muscle, binds tightly to many autonomic ganglia, it fails to block nicotinic transmission in most of these ganglia. Recently, we have isolated a second toxin, toxin F, that blocks transmission in several autonomic ganglia, including the chick ciliary ganglion. ^{125}I -Toxin F binds to 2 sites in the ciliary ganglion: one site that is also recognized by BGT and one site that is not. Since the presence of BGT fails to prevent the blocking effect of toxin F, the toxin F site not recognized by BGT most likely represents the neuronal nicotinic receptor. Accordingly, we have localized the binding of ^{125}I -toxin F to both sites, using electron-microscopic autoradiography.

After a 45 min incubation, ^{125}I -toxin F binding sensitive to BGT was primarily localized extrasynaptically on the neuronal plasma membranes; however, by 4 hr, much of this site had been internalized. In contrast, the ^{125}I -toxin F binding site not recognized by BGT was highly concentrated near synaptic membranes at both times. Pretreatment of ganglia with the classic nicotinic antagonists dihydro- β -erythroidine (DHBE) and α -tubocurarine (DTC) significantly reduced ^{125}I -toxin F binding to the toxin F-specific site. The average density of these sites on synaptic membranes was ~ 600 sites/ μm^2 .

We conclude that toxin F binds to 2 pharmacologically distinct sites in the ciliary ganglion and that these sites are distributed differently over the plasma membrane of ciliary neurons. On the basis of the density of the toxin F-specific binding sites at synaptic membranes, we infer that the density of synaptic nicotinic receptors on these neurons is at least 20-fold lower than the density of nicotinic receptors at the vertebrate neuromuscular junction, as determined by BGT binding. These findings are consistent with those of electrophysiological studies, which also suggest low nicotinic receptor densities on ganglionic neurons.

Unlike the case of nicotinic ACh receptors on skeletal muscle, little is known about the cellular distribution and density of

these receptors on neurons. This is due mainly to the lack of a suitable probe for such studies. Although α -bungarotoxin (BGT) binds with high affinity to sites on many neurons, in most cases BGT fails to block nicotinic function (for a review, see Oswald and Freeman, 1981). Thus, the use of BGT for the localization of neuronal nicotinic receptors is controversial. Autoradiographic studies of high-affinity binding sites for ^3H -nicotine and ^3H -ACh (in the presence of a muscarinic antagonist) have demonstrated an uneven regional localization of these sites in the central nervous system (e.g., Clarke et al., 1985); however, since these ligands are not fixed by standard electron-microscopic procedures, they are, at present, not appropriate for localization studies at the ultrastructural level.

We previously demonstrated that toxin F, a polypeptide snake toxin that, like BGT, is derived from the venom of *Bungarus multicinctus*, blocks nicotinic transmission in the chick ciliary ganglion (Loring et al., 1984). Furthermore, ^{125}I -toxin F binds to 2 sites in this ganglion, one of which is recognized by BGT and another of which is not. Since high concentrations of BGT do not prevent the pharmacological effect of toxin F, the toxin F-specific binding site is most likely to represent the locus of toxin F's action. The present study concerns the localization of ^{125}I -toxin F binding in chick ciliary ganglia, with particular emphasis on the localization of the toxin F-specific sites. Some of the results have been described in a preliminary communication (Loring and Zigmond, 1985).

Materials and Methods

Toxin F and BGT were purified from *Bungarus multicinctus* venom, as previously described (Lee et al., 1972; Loring et al., 1986). The toxins were iodinated by a modification of the chloramine T method (Hunter and Greenwood, 1962). Four to 20 μg toxin was dissolved in 100 μl of 0.15 M Tris-HCl buffer, pH 7.8, and mixed with 10 mCi Na^{125}I (low-pH, high-concentration Na^{125}I ; New England Nuclear, Boston, MA). Ten to 20 μg chloramine T in 5 μl Tris buffer was added and mixed well. Two minutes later, the reaction was stopped by adding 100 μl of 1 mg/ml sodium metabisulfite in water. Unreacted ^{125}I and damaged reaction products were separated from ^{125}I -labeled toxin by adding the reaction mix to a carboxymethylcellulose minicolumn (Whatman CM52; bed volume, 1 ml) that had been presaturated with 5 mM sodium phosphate buffer, pH 7.5, containing 1 mg/ml BSA. The column was washed with ten 1 ml washes of 5 mM sodium phosphate plus BSA, and ^{125}I -labeled toxin was step-eluted with five 1 ml washes of 50 mM sodium phosphate, pH 7.5, containing 0.3 M NaCl and 1 mg/ml BSA. Each wash was collected separately and diluted aliquots were counted. The initial specific activity of ^{125}I -toxin F ranged from 420 to 850 Ci/mmol. The initial specific activity of ^{125}I -BGT was 2600 Ci/mmol. The specific activity of ^{125}I -BGT was checked by an isotope dilution-binding experiment using membranes from *Torpedo* electric organ (Loring et al., 1982). The specific activity of ^{125}I -toxin F was similarly checked using

Received Oct. 2, 1986; revised Jan. 12, 1987; accepted Jan. 14, 1987.

We thank Jan Henderson and Wanda Hall for help in grain counting and measurement of membrane lengths and Dr. Jonathan Cohen for supplying membranes from *Torpedo* electric organ. This work was supported by U.S. Public Health Service Research Grant NS 12651 from the National Institutes of Health. R.E.Z. is supported by a Research Scientist Development Award (MH 00162).

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Figure 1. Time course of ^{125}I -toxin F binding to intact ciliary ganglia. Groups of 4 ciliary ganglia from 19 d *in ovo* embryos were incubated in 50 μl of Eagle's Minimal Essential Medium containing 1 mg/ml BSA and 10 nM ^{125}I -toxin F for the indicated times. Total (●) refers to binding in the absence of competing ligand. BGT (■) and TXF (▲) refer to binding in the presence of 2 μM α -bungarotoxin or 2 μM toxin F, respectively. Ganglia were incubated at 37°C in humidified 95% O_2 /5% CO_2 and then counted as described in Materials and Methods. Error bars represent the SEM. When not shown, the error was smaller than the symbol. *Inset*, Binding to the toxin F-specific site (counts in the presence of 2 μM α -bungarotoxin minus counts in the presence of 2 μM toxin F) plotted as a function of time. After a 4 hr incubation, the BGT-sensitive site bound 15 fmol ^{125}I -toxin F, while the toxin F-specific site bound 2.1 fmol.

^{125}I -toxin F binding to whole chick ciliary ganglia. Labeled toxins were stored at 4°C for not more than 6 weeks prior to being used.

Binding assays were performed essentially as described previously (Loring et al., 1985). Whole chick ciliary ganglia (from 17 to 19 d *in ovo* embryos; SPAFAS, Norwich, CT) were dissected, desheathed, and placed in 50 μl Eagle's Minimum Essential Medium (Gibco, Grand Island, NY) containing 1 mg/ml BSA. Preincubations with various drugs were carried out at 37°C for 1 hr in a humidified chamber containing 95% O_2 /5% CO_2 . Incubations with labeled toxin were carried out similarly, with the time of incubation ranging from 45 min to 4 hr. Electron-microscopic autoradiography was conducted as previously described (Loring et al., 1985), using the method of Salpeter and Bachmann (1964).

Results

We have previously demonstrated in radioligand-binding experiments that toxin F binds to 2 saturable sites in the chick ciliary ganglion, only one of which is also sensitive to BGT (Loring et al., 1984). In an attempt to establish equilibrium conditions for ^{125}I -toxin F binding to both sites, we incubated groups of 4 ciliary ganglia in 10 nM ^{125}I -toxin F for varying lengths of time, with and without the presence of either 2 μM BGT or 2 μM unlabeled toxin F (Fig. 1). Binding to the toxin F-specific binding site (i.e., binding in the presence of BGT minus binding in the presence of unlabeled toxin F) reached a plateau within 4 hr (inset, Fig. 1). In contrast, toxin F binding to the site affected by BGT (i.e., binding in the absence of any

Figure 2. Concentration dependence of ^{125}I -toxin F binding to intact ciliary ganglia. Groups of 4 ciliary ganglia from 19 d *in ovo* embryos were incubated for 4 hr in the presence of varying concentrations of ^{125}I -toxin F plus no competing ligand (●), 2 μM α -bungarotoxin (■), or 2 μM toxin F (▲). The BGT-sensitive site bound 14.5 fmol ^{125}I -toxin F, while the toxin F-specific site bound 3.3 fmol. Ratio of sites, 4.4:1.

competing ligand minus binding in the presence of BGT) continued to increase over the 4 hr period. ^{125}I -Toxin F binding to both sites is saturable and dose-dependent. As is shown in Figure 2, during a 4 hr incubation, both sites saturate at a concentration of approximately 10 nM ^{125}I -toxin F. The ratio of sites in this particular experiment is 4.4:1 (BGT-sensitive : toxin F-specific). A similar amount of binding to the BGT-sensitive site was obtained by measuring the binding of 20 nM ^{125}I -BGT to ciliary ganglia (Fig. 3). While the time course of toxin F binding to the BGT-sensitive site has not reached a plateau by 4 hr (Fig. 1), these sites are saturable at this time point at a concentration of 10 nM toxin F (Fig. 2). These data raise the possibility that additional BGT-sensitive sites become available during the course of the incubation.

In order to localize both ^{125}I -toxin F binding sites, ciliary ganglia from 17 d *in ovo* embryos were incubated either in 10 nM ^{125}I -toxin F for 45 min or in 20 nM ^{125}I -toxin F for 4 hr at 37°C. Groups of 4 ganglia were first preincubated in the presence of competing ligands for 1 hr and then incubated with ^{125}I -toxin F, as indicated in the legend to Figure 4. The BGT-sensitive site increased from 2.7 to 14.6 fmol/ganglion between the 45 min and 4 hr time points. The toxin F-specific site changed from 1.4 fmol/ganglion after 45 min to 2.3 fmol/ganglion after 4 hr (Fig. 4). To further characterize the pharmacological specificity of the toxin F-specific site, an examination of the ability of nicotinic agonists and antagonists to block ^{125}I -toxin F binding in the presence of 2 μM BGT was carried out. Dimethylphenylpiperazinium, a nicotinic agonist, and *d*-tubocurarine (DTC) and dihydro- β -erythroidine (DHBE), 2 nicotinic antagonists, all blocked the binding of toxin F (Fig. 4). The IC_{50} of the effects of the 2 antagonists was approximately 1 μM (data not shown). After the amount of binding was determined using gamma counting, certain ganglia from this experiment were prepared for electron-microscopic autoradiography.

Most of the specific autoradiographic labeling was found to be associated with neurons. There are 2 neuronal cell types in the chick ciliary ganglion, ciliary and choroid neurons. The ciliary neuron, at 17 d *in ovo*, has a large, ovoid cell body that is usually engulfed by a single large presynaptic terminal, termed

Figure 3. Time course of ^{125}I -BGT binding to intact chick ciliary ganglia. Groups of 4 ciliary ganglia from 19 d *in ovo* chick embryos were incubated for the indicated periods in 50 μl Eagle's Minimal Essential Medium containing 1 mg/ml BSA and 20 nM ^{125}I -BGT. Total (●) refers to binding in the absence of competing ligand; BGT (■) refers to binding in the presence of 2 μM BGT. After a 4 hr incubation, the specific BGT site bound 10.8 fmol ^{125}I -BGT.

the calyx. The smaller choroid neuron is innervated by multiple small nerve terminals from several preganglionic neurons, which synapse onto its cell body. (For more complete descriptions of the fine structures of these neurons, see Cantino and Mugnaini, 1975; Jacob and Berg, 1983; and Loring et al., 1985.) In the present study, only toxin F binding to ciliary neurons was examined.

In the absence of competing ligands (i.e., the combined labeling due to the BGT-sensitive site, the toxin F-specific site, and nonspecific binding), many regions of the neuron were so heavily labeled that detailed quantitative analysis was not possible, even following an autoradiographic exposure as short as 4 d. However, the following qualitative observations can be made. In ganglia exposed to ^{125}I -toxin F for 45 min, the majority of the autoradiographic grains were observed near the interface between the presynaptic nerve terminal and the cell body of the ciliary neuron (Fig. 5A). Much of the label was over regions of complex neuronal processes, an area we have previously identified as the major site of ^{125}I -BGT binding (Loring et al., 1985). Occasionally, a labeled cytoplasmic structure was encountered. In contrast, after a 4 hr incubation, most of the label was observed over cytoplasmic structures (Fig. 5B), which were often too heavily labeled to be identified. When cytoplasmic structures could be observed, lysosomes and multivesicular bodies were the most common organelles associated with clusters of grains. These data strongly suggest that a majority of the ^{125}I -toxin F binding sites are internalized between 45 min and 4 hr of incubation.

In the presence of 2 μM BGT (to remove the ^{125}I -toxin F site sensitive to BGT), many grains were observed near neuronal plasma membranes both after 45 min and 4 hr of incubation. A substantial portion of these grains was observed near specialized synaptic membranes (Fig. 6). Relatively few internal grains were observed in the presence of unlabeled BGT, as compared to the number observed in the absence of the toxin (Figs. 5, 6). Taken together with the data presented above, these results

Figure 4. Binding of ^{125}I -toxin F to chick ciliary ganglia: gamma counting of samples used for electron-microscopic autoradiography. Groups of 4 ciliary ganglia from 17 d *in ovo* embryos were incubated for 45 min in 10 nM ^{125}I -toxin F (A) or for 4 hr in 20 nM ^{125}I -toxin F (B). A: Bar A, no competing ligands; bar B, 2 μM BGT; bar C, 2 μM toxin F plus 2 μM α -BGT; bar D, 2 μM toxin F; bar E, 100 μM DHBE plus 2 μM BGT; bar F, 100 μM dimethylphenylpiperazinium plus 2 μM BGT. B: Bar G, no competing ligand; bar H, 2 μM BGT; bar I, 2 μM toxin F; bar J, 100 μM DTC plus 2 μM BGT; bar K, 100 μM DHBE plus 2 μM BGT. Ganglia were fixed and counted as described in the text. Selected ganglia were then processed for electron-microscopic autoradiography.

suggest that it is primarily the BGT-sensitive toxin F sites that are internalized.

In the presence of toxin F or DHBE, few grains associated with neurons were observed (Fig. 7). In order to quantitate these observations, the perimeters of randomly selected ciliary neurons were photographed and printed at 15,000 $\times$ magnification. All grains that lay within 0.25 μm of the neuronal plasma membrane were counted (Table 1). This area should contain ~80% of all grains arising from ^{125}I decays from a line source, such as a membrane (Salpeter et al., 1977). In addition, all grains that lay within 0.25 μm of specialized synaptic membranes were counted, and the lengths of those membranes were measured (see Loring et al., 1985, for details). On the basis of these values and on the exposure time, the specific activity of ^{125}I -toxin F, the sensitivity of the photographic emulsion, and the thickness of the section, the density of ^{125}I -toxin F binding sites per square micrometer of synaptic membranes was calculated (Table 1). Because of uncertainties in some of these values, the final densities should be regarded as estimates that could be off by a factor of 2 to 3. Also, the final values do not reflect the fraction of grains (~20%) originating from ^{125}I -toxin F bound to the plasma membrane but lying farther than 0.25 μm from the membrane.

Grains observed within 0.25 μm of synaptic membranes after a 45 min or a 4 hr incubation with ^{125}I -toxin F in the presence of BGT represented 38 and 55% of the total plasma membrane-associated grains. The density of these synaptic toxin F-specific binding sites was approximately 450–650/ μm^2 (Table 1). All ^{125}I -toxin F binding to synaptic membranes was blocked by treatment with 2 μM toxin F. Most (>90%) of the ^{125}I -toxin F binding at synapses was blocked by 2 μM BGT plus 100 μM DHBE; however, 2 μM BGT plus 100 μM DTC blocked only 50% of the binding. In addition to the presence of these toxin F-specific

Figure 5. Localization of bound ^{125}I -toxin F in the absence of competing ligands. In the absence of BGT, most of the ^{125}I -toxin F binding is to the BGT-sensitive site. *A*, A representative overexposed autoradiogram of a ciliary neuron after an incubation of 45 min in 10 nM ^{125}I -toxin F. Note the dense grain clusters near the surface of the neuron, particularly in regions referred to in the text as "complex" contact regions. *B*, An autoradiogram after a 4 hr incubation in 20 nM ^{125}I -toxin F. Much of the label has been internalized. Some grains, however, are still found near the surface of the neuron. *cb*, Ciliary neuron cell body; *s*, support cells; *nt*, nerve terminals. Magnification bars, 5 μm .

binding sites, the data suggest that some BGT-sensitive binding sites also are located at synapses. Thus, after a 4 hr incubation in the absence of BGT, the density of ^{125}I -toxin F binding to synapses was 960 sites/ μm^2 , a value approximately 300 sites/

μm^2 greater than that observed in the presence of BGT. Because of the intense labeling on plasma membranes of ganglia incubated for 45 min in the absence of BGT, and the possibility of radiation spread into synaptic membrane regions, the density

Table 1. Grain counts and synaptic densities of ^{125}I -toxin F binding to ciliary neurons

Incubation time	Competing ligand ^a	No. of ganglia analyzed	Grains within 0.25 μm of membrane	Grains within 0.25 μm of synapses	Synaptic area (μm^2)	Synaptic density ^b (sites/ μm^2)
45 min	None	0 ^c	—	—	—	—
45 min	2 μM BGT	3	302	116	15.6	479 $\pm$ 50
45 min	2 μM TXF + 2 μM BGT	3	21	0	12.1	—
45 min	100 μM DHBE + 2 μM BGT	1	5	1	3.9	17 $\pm$ 17
4 hr	None	3	575	129	14.1	959 $\pm$ 12
4 hr	2 μM BGT	3	168	93	15.2	638 $\pm$ 64
4 hr	2 μM TXF	3	15	0	10.5	—
4 hr	100 μM DHBE + 2 μM BGT	3	35	7	16.7	46 $\pm$ 16
4 hr	100 μM DTC + 2 μM BGT	3	101	34	12.3	319 $\pm$ 98

^a BGT, α -bungarotoxin; TXF, toxin F; DHBE, dihydro- β -erythroidine; DTC, *d*-tubocurarine.

^b Values represent the mean $\pm$ SEM of the values obtained from each ganglion or the autoradiographic error (Salpeter et al., 1977), whichever was greater. Thus the mean values may deviate slightly from those calculated on the basis of the total number of grains divided by the total membrane length.

^c Because of high grain densities, this group was not analyzed quantitatively (see text).

of binding for this group of ganglia could not be calculated.

In a previous study, we determined that synaptic membranes comprise only about 4% of the total membrane surface of ciliary neurons (Loring et al., 1985). The fact that, in the presence of BGT, 40–55% of the autoradiographic grains resulting from ^{125}I -toxin F binding to ciliary plasma membranes are found on about 4% of the total membrane suggests that, on the average, synaptic membranes are at least 10-fold more heavily labeled than membranes from other regions. To determine directly if this inference is true, we measured the length of all plasma membranes of the ciliary neurons that had been photographed. The categories of membranes tabulated were the same categories used in our analysis of ^{125}I -BGT binding to ciliary ganglia (Loring et al., 1985). Neuronal membranes that were not near presynaptic nerve terminals were classified as “noncontact” membrane regions. The contact regions between pre- and postsynaptic elements were further subdivided into “simple” and “complex” regions. “Simple” regions were those in which the presynaptic terminal and the cell body were in close apposition, with no intervening membranes. “Complex” regions were those in which membrane profiles were interposed between the major pre- and postsynaptic elements. Examples of these regions can be observed in Figure 6.

As in the earlier study (Loring et al., 1985), synaptic mem-

branes comprised between 4 and 5% of the neuronal surface. In addition, only about 20% of the total synaptic membrane area was found in “complex” regions. Grains within 0.25 μm of neuronal membrane were assigned to the nearest membrane category; these grain counts are tabulated in Table 2. From the grain counts and membrane lengths, we calculated the density of ^{125}I -toxin F binding sites for each membrane category. Synaptic densities of ^{125}I -toxin F binding sites (in the presence of 2 μM BGT) ranged from ~400 to 840 sites/ μm^2 , with somewhat higher values being observed on synaptic membranes in “complex” regions (Table 2). As predicted, nonsynaptic membrane densities were at least 10-fold lower than those of synaptic membrane (range, 11–39 sites/ μm^2). Again, somewhat higher values for nonsynaptic densities were found in “complex” regions.

Analysis of the distribution of grains over the cytoplasm of ciliary neurons was also performed (see Loring et al., 1985, for details). After a 45 min incubation with ^{125}I -toxin F in the presence of 2 μM BGT (to remove the BGT-sensitive site), the density of grains over the cytoplasm was about 4-fold higher than it was when unlabeled toxin F was included in the incubation (Table 3). After a 4 hr incubation, this ratio did not change significantly, although the absolute values increased. Both DHBE and DTC significantly reduced the internal label. Furthermore,

Table 2. ^{125}I -Toxin F binding to various regions of the plasma membrane of ciliary neurons (in the presence of 2 μM α -bungarotoxin)

Incubation time	Membrane regions	Total grains	Total membrane area (μm^2)	Sites/ μm^2 ^a
45 min	“Noncontact” membrane	12	64.4	11 $\pm$ 4
45 min	“Simple” nonsynaptic membrane	23	54.3	30 $\pm$ 7
45 min	“Simple” synaptic membrane	84	12.4	418 $\pm$ 81
45 min	“Complex” nonsynaptic membrane	151	249.7	39 $\pm$ 5
45 min	“Complex” synaptic membranes	32	3.1	665 $\pm$ 90
4 hr	“Noncontact” membrane	11	90.9	13 $\pm$ 6
4 hr	“Simple” nonsynaptic membrane	8	49.9	17 $\pm$ 3
4 hr	“Simple” synaptic membrane	71	12.6	577 $\pm$ 82
4 hr	“Complex” nonsynaptic membrane	56	170.7	34 $\pm$ 6
4 hr	“Complex” synaptic membrane	22	2.6	843 $\pm$ 114

^a Expressed as the mean $\pm$ SEM for the values obtained for each of the 3 ganglia analyzed at each time point. Thus the mean values may deviate slightly from those calculated based on the total number of grains divided by the total membrane length.

Figure 6. Electron-microscopic autoradiographs of ciliary neurons incubated in ^{125}I -toxin F for 45 min in the presence of $2\ \mu\text{M}$ BGT. Synaptic profiles are indicated by *black arrowheads*. In the presence of $2\ \mu\text{M}$ BGT (to occupy the BGT-sensitive binding site), autoradiographic grains were often observed within $0.25\ \mu\text{m}$ of postsynaptic membranes. Occasionally, grains located near synapses were found in clusters (e.g., *C*). Other grains were observed diffusely distributed near other neuronal plasma membranes (*small arrows*) and over neuronal cytoplasm (*white arrow*, *B*). The nonsynaptic grains indicated by *small arrows on the right-hand side of B* and by the *small arrow in C* are examples of grains found in "complex" contact regions. The nerve terminal and the cell body are separated in these zones by intervening membrane processes. The *leftmost small arrows in A and B* point to examples of grains found in "simple" contact regions that are not "synaptic." Thus, while the grains are within $0.25\ \mu\text{m}$ of the ciliary neuron plasma membrane and are in a region closely apposed to the presynaptic terminal, they lie further than $0.25\ \mu\text{m}$ from clearly identified synaptic profiles. *s*, Support cell; *cb*, ciliary neuron cell body; *nt*, nerve terminal. Calibration bars, $0.25\ \mu\text{m}$.

Figure 7. Inhibition of ^{125}I -toxin F binding by unlabeled toxin F or DHBE. In the presence of $2\ \mu\text{M}$ toxin F (A) or of $100\ \mu\text{M}$ DHBE plus $2\ \mu\text{M}$ BGT few autoradiographic grains were observed near neuronal plasma membranes. (B). Symbols as in Figure 6.

the grain density in the presence of these antagonists was not significantly different than the grain density in the presence of unlabeled toxin F.

Discussion

We have previously demonstrated that toxin F, a polypeptide isolated from *Bungarus multicinctus* venom, blocks nicotinic transmission in the chick ciliary ganglion (Loring et al., 1984)

and in the rat superior cervical ganglion (Sah et al., 1987). All of the available electrophysiological evidence indicates that the toxin acts postsynaptically, presumably at the level of the nicotinic receptor. Recent isoelectric focusing and amino acid sequence analysis (Loring et al., 1986) indicate that toxin F is identical to 2 toxins described by other laboratories: bungarotoxin 3.1 (Ravdin and Berg, 1979) and kappa-bungarotoxin (Chiappinelli, 1983). Thus, results obtained with these 2 other

Table 3. Density of ^{125}I -toxin F sites over neuronal cytoplasm

Incuba- tion time	Competing ligands	No. of ganglia ana- lyzed	μm^3 cytoplasm analyzed	No. of grains	Sites/ μm^3 ^a
45 min	BGT ^b	3	919	118	8.5 ± 1.6
45 min	TXF	3	870	29	2.1 ± 0.7
45 min	DHBE	1	276	15	3.5
4 hr	BGT	3	955	198	21.7 ± 0.9
4 hr	TXF	3	1049	64	6.4 ± 1.0
4 hr	DHBE	3	990	43	4.5 ± 0.8
4 hr	DTC	3	1004	93	9.7 ± 1.5

^a Expressed as the mean of values for each ganglia $\pm$ SEM.^b Concentrations of competing ligands used in each group are given in Table 1.

toxins are directly applicable to the present study. Unlike toxin F, BGT fails to block nicotinic function in the chick ciliary ganglion (e.g., Chiappinelli et al., 1981). Curiously, ^{125}I -BGT does bind with high affinity to sites in this ganglion, and this binding is blocked by a variety of nicotinic agonists and antagonists (e.g., Chiappinelli et al., 1981). However, electron-microscopic studies indicate that the binding of BGT in the ciliary ganglion is primarily extrasynaptic (Jacob and Berg, 1983; Loring et al., 1985). In addition, a saturating concentration of BGT fails to prevent the pharmacological effect of either toxin F (Loring et al., 1984), bungarotoxin 3.1 (Ravdin et al., 1981), or kappa-bungarotoxin (Chiappinelli, 1983). These data suggest that BGT does not bind to synaptic nicotinic receptors on chick ciliary neurons.

We have previously reported (Loring et al., 1984) that ^{125}I -toxin F binds to 2 sites on chick ciliary ganglia: one site that is also recognized by BGT, and one site that is not. Similar results have been reported for ^{125}I -kappa-bungarotoxin (Chiappinelli, 1983). It was, therefore, of interest to determine the localization of both of these toxin F binding sites.

After a 45 min incubation, most of the BGT-sensitive ^{125}I -toxin F binding sites in the ciliary ganglion were found near the plasma membrane of ciliary neurons, particularly over complex contact regions. Because of the heavy labeling, it was not possible to measure the density of these binding sites. However, the distribution of these BGT-sensitive ^{125}I -toxin F sites is qualitatively similar to the distribution of ^{125}I -BGT binding sites in this ganglion, as we previously reported (Loring et al., 1985). In our previous study, over 70% of the autoradiographic grains resulting from the binding of ^{125}I -BGT were observed in complex contact regions, while less than 3% of the grains were observed near synaptic membranes (Loring et al., 1985). Furthermore, the density of BGT binding was fairly uniform over the surface of ciliary neurons, ranging from 100 to 250 sites/ μm^2 . In addition, about 20% of the autoradiographic grains were observed over neuronal cytoplasm. On the basis of these densities, the surface area, and the cytoplasmic volume of a ciliary neuron (Cantino and Mugnaini, 1975), we estimated, in our previous study, that a single ciliary neuron binds about 3.8×10^6 BGT molecules. It is of interest that, in the present study, the majority of BGT-sensitive ^{125}I -toxin F sites were over cytoplasmic areas after a 4 hr incubation. Ravdin et al. (1981) have demonstrated, using labeled BGT, that bungarotoxin 3.1 causes a time-dependent internalization of BGT bound to cultured neurons from chick ciliary ganglia. Their data suggest that the site of inter-

action between bungarotoxin 3.1 and BGT is the BGT binding molecule. The present study indicates that toxin F is also internalized when bound to the BGT molecule.

In the presence of ^{125}I -toxin F and BGT (in order to detect only the toxin F-specific sites), as many as 55% of the autoradiographic grains near surface membranes were found near synapses (Table 1). The average density of the ^{125}I -toxin F-specific binding site is ~ 600 sites/ μm^2 at synaptic membranes and ~ 20 sites/ μm^2 on extrasynaptic membranes (Table 2). In addition, about 45% of the specific label is found over neuronal cytoplasm (Table 3). On the basis of these densities, the relative surface membrane areas, and the cytoplasmic volume of ciliary neurons, we estimate that a single ciliary neuron binds about 1.2×10^6 toxin F molecules at toxin F-specific sites. Thus, we calculate that the ratio of total BGT binding sites to toxin F-specific binding sites per neuron should be about 3.2:1. This value falls near the lower range of ratios we have observed for ^{125}I -toxin F binding to these 2 sites, the actual ratio being dependent on the time of incubation and other factors. It should be emphasized that the "toxin F-specific" binding site is not an artifact due to incomplete saturation of BGT binding sites by $2 \mu\text{M}$ unlabeled BGT. Thus, increasing the concentration of BGT to $50 \mu\text{M}$ had no effect on the binding of ^{125}I -toxin F to the toxin F-specific site. Furthermore, $50 \mu\text{M}$ BGT does not prevent the pharmacological blocking effect of a 1000-fold lower concentration of toxin F (Loring and Zigmond, unpublished observations). Finally, it should be noted that this study deals with embryonic ganglia. While the number of BGT binding sites in the ciliary ganglion reaches adult levels by about 11 d *in ovo* (Chiappinelli and Giacobini, 1978), we do not know the developmental time course for toxin F-specific binding at synapses.

We reported previously that neither the nicotinic antagonist DTC nor the agonist carbachol inhibited ^{125}I -toxin F binding to the toxin F-specific site, as measured by gamma counting of whole ganglia (Loring et al., 1984). In contrast, in the present study we found that DHBE, DTC, and dimethylphenylpiperazine block the binding of ^{125}I -toxin F to the toxin F-specific site. Furthermore, we have recently found that carbachol also blocks this binding (Loring and Zigmond, unpublished observations). We do not know the reason for the difference between the 2 studies; however, one important difference is the higher purity of the ^{125}I -toxin F we are currently using (Loring et al., 1984, 1986). Given the fact that DTC was as effective as DHBE and unlabeled toxin F in blocking total toxin F-specific binding, as measured by gamma counting, it is somewhat puzzling that this antagonist only blocked about 50% of the ^{125}I -toxin F binding at synaptic membranes of ciliary neurons. If DTC were more effective at blocking toxin F binding at other sites in the ganglion (as it seems to be for the internalized binding sites), the failure of DTC to completely block a subset of toxin F-specific binding sites (i.e., those at ciliary synaptic membranes) might be difficult to detect by gamma counting.

The pharmacology and ultrastructural localization of the toxin F-specific binding sites on ciliary neurons, together with our earlier electrophysiological studies (Loring et al., 1984), suggest that these sites are the nicotinic receptors of ciliary neurons. It is interesting that the amount and distribution of ^{125}I -toxin F binding to the toxin F-specific site are strikingly similar to the distribution and amounts of binding seen in ciliary ganglia using monoclonal antibodies raised against the nicotinic receptor of the eel electric organ (Jacob et al., 1984). These monoclonal

antibodies recognize the main immunogenic region of the α -subunit of the ACh receptor isolated from the electric organ, and they do not recognize the BGT binding site on ciliary ganglion neurons (Smith et al., 1985). Smith et al. (1986) reported that 8 d embryonic ciliary ganglion neurons maintained in culture for 7 d have about 2 fmol of surface monoclonal antibody binding sites per ganglion, a number very similar to the 2–3 fmol of ^{125}I -toxin F specific binding sites we find in intact 17–19 d embryonic ganglia (Figs. 1, 2, 4). Furthermore, using electron-microscopic immunoperoxidase techniques, Jacob et al. (1984, 1986) demonstrated that the monoclonal antibodies bind primarily to synaptic regions on embryonic ganglia, although occasionally small, nonsynaptic patches of labeling are observed in regions that we refer to as “complex” contact zones. The present study demonstrates high concentrations of ^{125}I -toxin F binding (in the presence of BGT) at synaptic sites. While it would be extremely difficult for the autoradiographic technique to pick up small, discrete patches of ^{125}I -toxin F binding to nonsynaptic complex contact regions, we do observe large numbers of grains and the highest nonsynaptic site densities of ^{125}I -toxin F binding in the regions where nonsynaptic antibody patches were observed. These results strongly suggest that toxin F and the monoclonal antibodies recognize the same component of ciliary neuron membranes.

Of the membrane-associated toxin, about half was found near synaptic membranes, which represent only 4–5% of the total neuronal surface. We calculate that the density of binding sites is about 10–20-fold higher at synapses than on other membranes (Table 2). This distribution is similar to that of ACh sensitivity found on frog parasympathetic neurons. In one of the few studies on neuronal nicotinic receptor distribution, Harris et al. (1971) observed variations in ACh sensitivity of 10–20-fold over the surface of innervated neurons in the frog heart septum. ACh iontophoresed close to synaptic boutons produced large depolarizing responses that peaked more rapidly than similar responses at nonsynaptic regions, which suggested to the authors that the ACh had to diffuse some distance to reach the higher receptor densities at synapses. This distribution of receptors is markedly different than that found at the neuromuscular junction of innervated vertebrate muscle, where the synaptic membrane is 50–100-fold more sensitive to ACh than is the extrasynaptic membrane (e.g., Kuffler and Yoshikami, 1975) and the BGT binding density is 500–1000-fold higher at the junction than elsewhere (e.g., Fertuck and Salpeter, 1976). Distributions of ACh sensitivity similar to that on frog cardiac ganglion neurons have also been described for mudpuppy cardiac ganglia (Roper, 1976) and bullfrog sympathetic ganglia (Dunn and Marshall, 1985). Thus, the distribution of neuronal receptors on chick ciliary ganglion neurons, as inferred from the binding of toxin F, is consistent with electrophysiological data from other autonomic ganglia.

The available electrophysiological data, although subject to alternative interpretations, also suggest that the density of synaptic receptors in autonomic ganglia is substantially lower than that at the neuromuscular junction. One functional difference between the vertebrate neuromuscular junction, which is found on long, cylindrical muscle cells, and synapses on ciliary neurons, which are found on smaller, spherical cell bodies, is the higher input resistance at the latter (e.g., Harris et al., 1971). Thus, smaller synaptic currents should be necessary for bringing ciliary neurons to threshold. Ogden et al. (1984) have measured the ACh receptor channel properties of ciliary neurons and found

them to be comparable to those of receptors at the neuromuscular junction. The number of channels opened by a single quantum of ACh has been measured in several autonomic ganglion neurons [i.e., the rat submandibular ganglion (Rang, 1981), the bullfrog sympathetic ganglion (L. Marshall, personal communication), the rabbit superior cervical ganglion (Derkach et al., 1983), and the chick ciliary ganglion (Dryer and Chiappinelli, 1987)] and has been found to be between 75 and 150 channels/quantum. In contrast, a single quantum opens about 1000–2000 channels at the frog neuromuscular junction (Katz and Miledi, 1972; Anderson and Stevens, 1973). Thus, the synaptic currents at neuronal synapses appear to be limited by the reduced number of open receptor channels. As discussed by Rang (1981), the decreased number of ACh channels opened on neurons may reflect a reduced number of receptors at neuronal synapses or a lower affinity of the neuronal receptor for ACh. Other explanations, such as lower amounts of ACh released per quantum or increased concentrations of AChE at the neuronal synapse, can probably be discounted (Rang, 1981). Ogden et al. (1984) have found evidence for both low receptor densities and reduced receptor affinity for agonists in cultured chick ciliary neurons. Given the number of unknown variables, predictions of an absolute density of nicotinic receptors on autonomic neurons based solely on electrophysiological data would be premature. Nevertheless, a reduced density of postsynaptic receptors remains one possible explanation for the reduced number of opened channels per quantum in autonomic neurons, compared to the number at the neuromuscular junction. The data in the present study strongly suggest that the density of nicotinic receptors at ciliary neuron synapses is at least an order of magnitude lower than the receptor density at the neuromuscular junction.

In addition to blocking nicotinic transmission in the chick ciliary ganglion, toxin F has been found to cause a slowly reversible blockade of nicotinic function in other autonomic neurons, such as cultured neurons derived from the rat superior cervical ganglion (Sah et al., 1987). Thus, toxin F should serve as a useful probe to localize neuronal nicotinic receptors in these preparations as well. Experiments are currently in progress to determine whether toxin F blocks nicotinic responses in the central nervous system. If so, the toxin should prove useful in characterizing neuronal nicotinic receptors more generally.

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