involves both hydrophobic and polar regions of the scorpion toxin.

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Registry No. Li, 7439-93-2; Ca, 7440-70-2; Mg, 7439-95-4; Mn, 7439-96-5; guanidinium, 25215-10-5.

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Properties of Curaremimetic Neurotoxin Binding Sites in the Rat Central Nervous System†

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ABSTRACT: Properties of mammalian central nervous system binding sites for curaremimetic neurotoxins are investigated with the Simonsen-Albino rat and ^{125}I-labeled α-bungarotoxin or the principal neurotoxin from *Naja naja siamensis*. Evidence is presented that high-affinity toxin binding sites are distributed as expected for a synaptic neurotransmitter receptor, display distinct nicotinic cholinergic pharmacology, and are sensitive to preincubation with nicotinic agonists. Affinity of toxin sites for agonists is altered by specific sulfhydryl/disulfide modification and by Ca^{2+}, and sites may be labeled with the nicotinic acetylcholine receptor affinity reagent bromoacetylcholine. New data are also presented indicating that toxin binding sites with $\bar{K}'$ values of ~1 nM and ~100 nM may be detected on rat brain crude mitochondrial fractions. Evidence is also reported suggesting the existence of two classes of toxin binding site disulfides/sulfhydryls, which interact with affinity reagents and nonspecific alkylating agents and are located proximal and distal, respectively, to the acetylcholine binding site. The results indicate that central nervous system (CNS) toxin binding sites share significant biochemical homology with nicotinic receptors from the periphery and provide a foundation for further study of toxin binding site biochemistry and the relationship between toxin sites and authentic CNS nicotinic acetylcholine receptors.

The introduction of curaremimetic neurotoxins as neuromuscular junction nicotinic receptor ligands was predicated upon demonstration of their antagonistic potency at that locus (Lee, 1972). Subsequent molecular characterization of nicotinic receptors from the electric organ of ray and eel relied heavily on application of snake neurotoxins (Karlin, 1980; Conti-Tronconi & Raftery, 1982).

Since the identification of acetylcholine as a neurotransmitter substance at the Renshaw cell [see Curtis & Eccles

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(1958)], it has been of interest to characterize central nervous system (CNS)[1] cholinergic transmission in molecular detail, particularly by comparison to neuromuscular junction synaptic mechanisms. Neurotoxins potent at the neuromuscular junction offered promise as probes for identification of CNS acetylcholine receptors (AcChoR). Binding sites for α-bungarotoxin (Bgt), for example, in the mammalian CNS show properties and distribution as expected for a CNS AcChoR [see Morley & Kemp (1981)]. However, this radioreceptor assay work was periled by evidence showing relative impotency of Bgt as an antagonist at presumptive autonomic and CNS cholinergic synapses [see Schmidt et al. (1980)].

Our earlier work with rats from the Wag/Rig strain was intended to provide support for biochemical homology between CNS Bgt binding sites and AcChoR from the periphery and for the existence of certain CNS Bgt binding site properties that could be correlated with aspects of true AcChoR function (Lukasiewicz & Bennett, 1978; Lukas & Bennett, 1979a,b, 1980a,b; Lukas et al., 1979). Our current goal is to develop integral model systems, with Simonsen-Albino rats, that permit continued study of curaremimetic neurotoxin binding sites as well as investigation of authentic CNS AcChoR function. In order to establish a foundation for such studies, we report here new data confirming and extending our earlier observations regarding the properties of Bgt binding sites in the rat CNS. A preliminary account of some of this work has already appeared (Lukas, 1981).

Experimental Procedures

Bgt Purification and Iodination. Purification of Bgt is achieved at 4 °C via modification of previously published procedures (Eterovic et al., 1975; Lukasiewicz et al., 1978). Crude *Bungarus multicinctus* venom (Miami Serpentarium) is fractionated via gradient elution (1.5 L of 50 mM NH_4OAc, pH 5.0, to 500 mM NH_4OAc, pH 7.0, then 0.8 L of 500 mM to 1 M NH_4OAc, pH 7.0) on CM-cellulose CM-52 (Whatman). The fraction identified as peak 3 is subjected to gel-filtration chromatography on Bio-Gel P-30 (Bio-Rad) equilibrated in 0.1 M NH_4OAc, pH 6.8, 4 °C. The principal, low molecular weight fraction is lyophilized, desalted on Bio-Gel P-2 in 25 mM NH_4OAc, pH 6.0, 4 °C, and isolated on CM-Sepharose CL-6B (Sigma) via gradient elution, 800 mL of 25 mM NH_4OAc, pH 6.0, to 300 mM NaCl–25 mM NH_4OAc, pH 6.0. Material in the single eluted fraction is lyophilized, desalted, lyophilized, and stored at −20 °C as purified Bgt.

Radioiodinated Bgt is prepared at room temperature in a fume hood as described earlier (Lukasiewicz et al., 1978) except that column buffers are adjusted to pH 6.0, and unlabeled and ^{125}I-labeled Bgt species are resolved via gradient elution, 400 mL of 5 mM Na_2HPO_4, pH 6.0, and 0–200 mM NaCl, 4 °C. A more detailed description of Bgt purification and iodination is found in the supplementary material (see paragraph at end of paper regarding supplementary material).

Najatoxin Purification and Iodination. Crude venom from *Naja naja siamensis* (Miami Serpentarium), manipulated as indicated above for Bungarus venom, is resolved into multiple components essentially as described by Karlsson et al. (1971), but on CM cellulose CM-52 ion-exchange resin and with a linear gradient from 0.14 to 1.4 M NH_4OAc, pH 6.5, 1.5-L total volume. The principal neurotoxin (najatoxin) is purified by gel-filtration and ion-exchange chromatography and stored in low-salt, lyophilized form, as described above for purification of Bgt.

Iodinated najatoxin (I-najatoxin) is prepared by using a chloramine T method (Greenwood et al., 1963). To a 90-μL solution containing 2 mCi of ^{125}I (carrier free, low pH), 51 nmol of KI, and ~40 nmol of najatoxin in 20 mM Na_2HPO_4, pH 7.4, at 20 °C is added 10 μL of 1.15 mg/mL chloramine T in 0.1 M Na_2HPO_4, pH 7.4. After 5 min, the reaction mixture is applied to a Bio-Gel P-2 column to resolve labeled product from free iodine, as above for iodinated Bgt. The void volume fraction is loaded onto a CM-cellulose CM-52 column at 4 °C and eluted as also described above.

Preparation of Torpedo Electroplax Membranes. AcChoR-rich membranes from frozen electric organ of *Torpedo californica* (Pacific Biomarine) are prepared, with minor modifications, essentially as described by Hazelbauer & Changeux (1974) and are resuspended in dilute buffer to a concentration of 2–8 μM I-Bgt sites for storage at 4 °C.

Characterization of Radiolabeled Toxins. Procedures for characterizing radiotoxin preparations generally followed those described by Blanchard et al. (1979) and Lukas et al. (1981), with the exception that the DE-81 disc assay (Schmidt & Raftery, 1973) is used for all *Torpedo*/radiotoxin binding assays.

Rat Brain Crude Mitochondrial Fractions. Crude mitochondrial fractions are prepared fresh daily from brain (less cerebellum) of young adult male S/A Simonsen-Albino (SD) Fbr rats essentially as described previously (Lukas et al., 1979), except that they are recovered as pellets following centrifugation of crude nuclear supernatants at 35000*g* for 10 min (Sorvall RC-5B, SM-24 rotor). Samples are then suspended in the appropriate buffer, sedimented at 35000*g* for 10 min, resuspended in the appropriate buffer (Lukas et al., 1979), and either divided into aliquots for initiation of binding assays or subjected to chemical modification as described below.

General Outline of Assay Protocols.[2] Initial measurements of the extent of specific radiotoxin binding in every case are determined as the difference in radioactivity bound to test samples chased with a 100-fold excess (over radiotoxin) of native toxin for 5–15 min following incubation with radiolabeled toxin and blank samples preincubated with native toxin prior to addition of labeled toxin. The native toxin chase period serves to terminate specific radiotoxin binding and also permits equilibrium to be reached, with respect to nonspecific and short-lived pseudospecific binding, in both test and blank samples. In experiments where empirical evidence indicates that there is no significant difference in the data for native toxin chased test samples and test samples not subjected to native toxin chase, the chase paradigm is followed only intermittently thereafter, resulting in conservation of purified toxin. In the experiments reported below, the only instance in which the chase paradigm is followed routinely is in generation of toxin saturation curves, where a small but reproducible difference in chased and nonchased samples is evinced. Since the emphasis in those experiments is to examine evidence for low-affinity specific toxin binding components, it is critically important to control for nonspecific binding-derived artifacts [see Lukas (1984)]. Toward that end, the native toxin

[1] Abbreviations: CNS, central nervous system; AcChoR, nicotinic acetylcholine receptor; Bgt, α-bungarotoxin; NH_4OAc, ammonium acetate; I-Bgt, ^{125}I-labeled monoiodinated α-bungarotoxin; najatoxin, the principal curaremimetic neurotoxin from *Naja naja siamensis*; I-najatoxin, ^{125}I-labeled iodinated najatoxin; NbS_2, 5,5′-dithiobis(2-nitrobenzoic acid); IC_{50}, concentration of drug giving 50% inhibition of toxin binding.

[2] An earlier publication (Lukas et al., 1979) should be consulted for derivation of equations describing slowly reversible binding of a toxin to specific binding sites under preequilibrium conditions. The theoretical treatment provides definitions for the descriptive constants used throughout this paper, addresses modification of toxin binding in the presence of competitors, and provides justification for the graphical representations and analyses of data that are presented herein.

chase ensures that the final concentrations of labeled and unlabeled toxin and the final specific radioactivity of ligand are equal in blank and test samples. For the other experiments, performed at a fixed concentration of radiotoxin (unless otherwise noted, 10 nM in a 250-μL reaction volume), specific binding levels under chase and nonchase paradigms are equivalent following the first cycle of dilution, centrifugation, and resuspension (see below), consistent with efficient removal of free toxin species by this method.

In every case, I-Bgt binding assays are terminated by 10–12-fold dilution of assay sample in incubation medium and centrifugation at 40000g for 10 min in the Sorvall SM-24 rotor (4 °C). Membrane pellets are subjected to two more cycles of suspension in fresh buffer (3 mL) and sedimentation prior to final resuspension in 0.1 M NaOH–0.1% sodium dodecyl sulfate for transfer to γ-counting tubes for ^{125}I detection (70% efficiency). I-Najatoxin binding assays are terminated by three cycles of dilution/suspension with 1 mL of buffer and centrifugation for 5 min in a Beckman microfuge prior to direct counting of assay tubes containing the final pellet. All toxin binding assays are carried out with shaking at 20 °C. Radiotoxin titration experiments, studies of radiotoxin dissociation from toxin–toxin binding site complexes (formed following a 1-h, rather than an overnight, incubation at 10 nM I-Bgt), and toxin association studies are as described (Lukas et al., 1981).

Density gradient centrifugation and solubility studies were carried out on bulk prepared test and blank (native toxin preincubation) samples. Samples were labeled at 10 nM I-Bgt for 1 h and washed free of unbound toxin by two to three cycles of suspension and sedimentation as described above prior to final resuspension in the appropriate buffer. Sedimentation velocity and phase partitioning experiment fractions were assayed in 500-μL and 1.1-mL reaction volumes, respectively, as described above. Ionic strength experiment assays were performed as described above, but in solutions modified as outlined in the appropriate figure legend. Synaptic plasma membranes and synaptic junctional complexes were prepared according to Cotman & Taylor (1972) and Davis & Bloom (1973) following or prior to labeling with I-Bgt as described above.

Sulfhydryl/disulfide modification protocols are as described elsewhere (Lukas et al., 1979). As used below, the term "treatment" shall refer to reaction of membranes with chemical agents followed by a dilution and centrifugation cycle. The sequential treatment of membranes with dithiothreitol, followed by dilution, centrifugation, and treatment with N-ethylmaleimide, for instance, shall be abbreviated as dithiothreitol–N-ethylmaleimide, where the dash represents insertion of a dilution and centrifugation step. In instances where a particular treatment commences following preincubation of membranes with cholinergic ligand, and ligand is not removed by dilution and centrifugation, the term "preincubation" will be used, and a slash shall indicate the sequence of reaction. For example, preincubation with acetylcholine at 200 μM followed by addition of dithiothreitol, dilution, centrifugation, and treatment with N-ethylmaleimide shall be abbreviated as 200 μM acetylcholine/dithiothreitol–N-ethylmaleimide.

Preincubation and coincubation (where radiotoxin and ligand are mixed together prior to initiation of binding assay by addition of membranes) ligand competition experiments are as detailed elsewhere (Lukas et al., 1979) except that the duration of exposure to radiotoxin is for 60 min. Affinity reagent preparation and use are as previously described (Lukas & Bennett, 1980b), except that two cycles of dilution and centrifugation are used to terminate the reaction and ensure

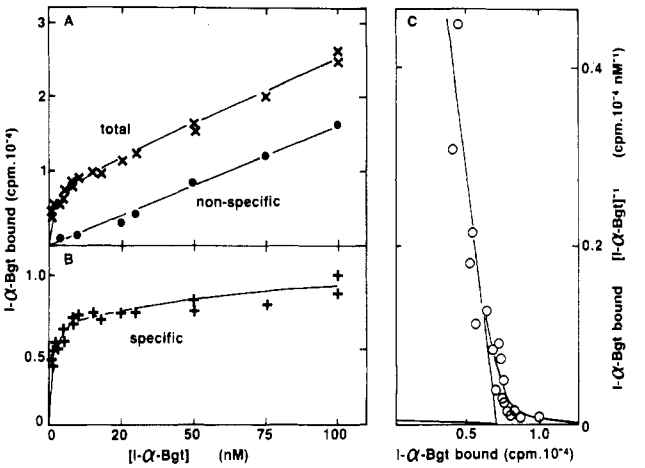

FIGURE 1: I-Bgt binding saturation profiles. Data points for total [(A) (×) test samples chased with excess native Bgt], nonspecific [(A) (●) blank samples preincubated with native Bgt], and specific [(B) (+) test minus blank samples) I-Bgt binding to rat brain crude mitochondrial fractions are plotted in linear fashion against final I-Bgt concentration ([T]) for reactions in an assay volume of 250 μL. Also plotted (C) is a modified Hofstee–Eadie–Scatchard-type representation of specific toxin binding data (○). Solid lines in (B) and (C) that are fit to the data by reiterative methods represent theoretical curves[2] conforming to the equation $v = V'_1[T](\bar{K}'_1 + [T])^{-1} + V'_2[T](\bar{K}'_2 + [T])^{-1}$ where v is the observed quantity of specific toxin binding and V'_1 and $\bar{K}'_1$ and V'_2 and $\bar{K}'_2$ are the apparent binding levels at 100% saturation and apparent dissociation constant for toxin interaction with toxin binding sites 1 and 2, respectively. Parameters used in generating the theoretical curves are $V'_1 = V'_2 = 7000$ cpm, $\bar{K}'_1 = 1$ nM, and $\bar{K}'_2 = 200$ nM. Membrane concentration is about 19 mg/mL, I-Bgt specific activity is 53 cpm/fmol, and data points are reproducible within a standard error of less than 10%.

quantitative removal of affinity ligand. Note that the same nomenclature and abbreviation style will be used to describe affinity reagent experiments. For example, dithiothreitol treatment followed by preincubation with acetylcholine and addition of bromoacetylcholine shall be abbreviated as dithiothreitol–acetylcholine/bromoacetylcholine.

Membrane protein concentrations are ordinarily determined by a modified Lowry et al. (1951) procedure, where samples are dissolved in NaOH–bicarbonate supplemented with 1% sodium dodecyl sulfate, and indexed to a bovine serum albumin standard.

Results

I-Bgt Binding. By utilization of fresh preparations of I-Bgt, and a nonequilibrium membrane centrifugation assay, specific binding of toxin to Simonsen-Albino rat brain crude mitochondrial fraction membranes is observed (Figure 1). Binding is dominated by a high-affinity (preequilibrium $\bar{K}'_1 \simeq 1$ nM) component at a concentration of approximately 28 fmol/mg of membrane protein. Upon closer examination, it is apparent that a second, low-affinity Bgt binding component is expressed at higher radiotoxin concentrations. Modified linear and Hofstee–Eadie–Scatchard-type plots of specific binding data are well fit to a theoretical curve on the basis of a 1:1 stoichiometry between high- and low-affinity sites, with $\bar{K}'$ values of 1 and 200 nM, respectively. A more detailed characterization of low-affinity I-Bgt binding is the subject of a study to be published elsewhere (Lukas, 1984). The bulk of the data in this paper is devoted to characterization of the high-affinity Bgt binding component. At the concentrations of radiotoxin used in the experiments reported below, no more than 10% of the observed binding represents interaction of I-Bgt with the low-affinity component.

In Figure 2 is illustrated kinetic properties of the interaction of I-Bgt with high-affinity binding sites in the rat CNS.

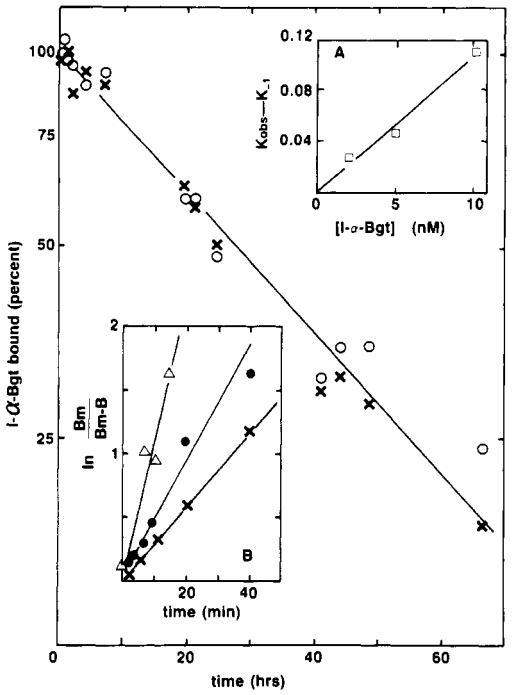

FIGURE 2: I-Bgt binding kinetics. Radiotoxin dissociation on physical (O) and chemical (×) dilution is plotted as percent of initial binding (log scale) as a function of time of dilution. Toxin association rate data are plotted as ln [(sites bound at 12 h)(sites bound at 12 h − sites bound at time t)$^{-1}$] against time (insert B). The observed rate of toxin association less the value for the dissociation rate constant, k_{-1}, is plotted (insert A) as a function of toxin concentration to yield a line with slope = k_1.

Radiolabeled toxin dissociation from binding sites fits a single exponential curve, with an apparent half-life of 27.5 h, yielding $k_{-1} = 4.2 \times 10^{-4}$ min^{-1}. Note that the toxin dissociation constant is essentially the same for samples treated by chemical or physical dilution. Analysis of association of Bgt with high-affinity toxin sites (Figure 2, inserts a and b) yields a value for k_1 of 1.1×10^{-2} min^{-1} nM^{-1}. The resultant microscopic K_D (k_{-1}/k_1^{-1}) for I-Bgt binding (~0.04 nM) is lower than the $\bar{K}'$ value for the high-affinity site obtained on 1-h incubation with I-Bgt. This predictable result is partly a consequence of the slow rate of toxin association, which is exacerbated at low concentrations of radiolabel. $\bar{K}'$ values decrease as the incubation time for I-Bgt titration curves increases (data not shown), and true equilibrium conditions are more closely approached.

Physical Properties of I-Bgt Binding Sites. Data describing the distribution of I-Bgt binding sites, fractionated by differential sedimentation, are shown in Table I. Approximately 44% of I-Bgt binding sites are present in conventionally defined crude mitochondrial fraction cuts (2000–12 000 g), while approximately 33 and 23% of toxin sites are present in crude nuclear (0–2000 g) and microsomal (12 000–100 000 g) fractions, respectively. Crude fractions defined in these terms each contain about one-third of total protein. By making a modification so that crude "mitochondrial" fraction membranes are taken from a 2000–35 000-g cut, greater than 60% of toxin binding sites, and less than 50% of total protein, may be recovered. This cut includes two fractions with the highest specific binding, and by repetition of the homogenization step on crude nuclear pellets, recovery of toxin binding sites is further increased in the modified mitochondrial fraction used in these studies.

Subcellular fractionation of crude mitochondrial fractions (2000–35 000-g cut) by the methods of Cotman & Taylor (1972) and Davis & Bloom (1973) yields synaptic plasma membranes and synaptic junctional complexes that are enriched 2- and 4-fold, respectively, in Bgt binding sites relative to starting material (identical results are obtained for samples prelabeled with radiotoxin or for samples labeled with toxin after purification), but with less than 20% yield.

Table I: Sedimentation Velocity Profile for I-Bgt Binding Sites[a]

g_{max}	cpm/assay	mg/mL	cpm/mg
389	9500	3.25	6640
1 596	12200	3.25	8520
3 580	12650	2.05	14020
6 360	8500	2.18	8860
12 000	7400	1.82	9230
20 900	6500	1.00	14770
30 400	3300	1.63	4600
39 700	1600	1.09	3333
60 000	1200	1.02	2670
120 000	800	0.77	2080
200 000	500	0.87	1300
317 500	1000	0.67	3390

[a] Sucrose (0.32 M) homogenates of rat brain were subjected to differential sedimentation at the indicated centrifugal force for 10 min at 4 °C. Pellets obtained at the indicated g force were pooled and assayed for I-Bgt binding (centrifugation assay as described under Experimental Procedures except that pellets obtained at 60000g or greater were centrifuged in the A-321 rotor and IEC B-60 centrifuge), while supernatants were subjected to further centrifugation. Assay volume was 500 μL containing 440 μL of membranes.

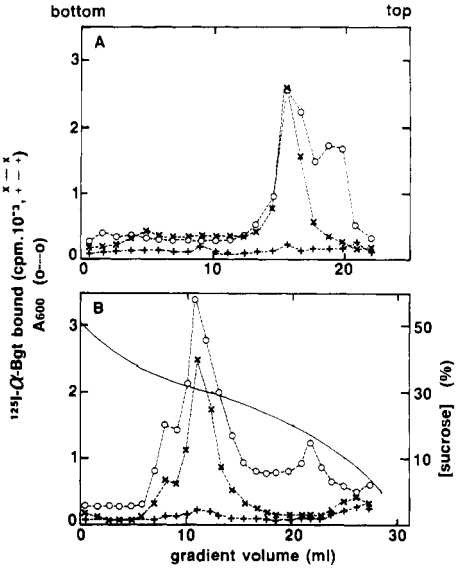

FIGURE 3: Density gradient profiles of I-Bgt binding sites. Crude mitochondrial fraction membranes are labeled at 10 nM I-Bgt as described under Experimental Procedures and resuspended in Ringer's buffer. Aliquots of 2.68 mL of test or blank samples are loaded onto a discontinuous sucrose gradient comprised of 1 mL of 10%, 7.5 mL of 22%, 7.5 mL of 30%, 7.5 mL of 40%, and 1.5 mL of 50% sucrose in Ringer's buffer in a swinging-bucket rotor (Model SB-110) and centrifuged at 18 000 rpm for 15 h in the IEC B-60 centrifuge (Figure 6B). Aliquots of 2.51 mL are mixed with 5 mL of isoosmolar Percoll (prepared in Ringer's buffer) and 15 mL of Ringer's buffer (yielding a final Percoll density of 1.0406 g/mL) and subjected to centrifugation at 14 500 rpm for 30 min in the Sorvall SM-24 rotor (Figure 6A). Plotted against gradient volume are I-Bgt bound in test (×) and blank (+) sample fractions and approximate membrane protein concentration [(O); measured as scattering absorbance at 600 nm for Percoll gradients and absorbance at 600 nm for 1-mL sucrose gradient fractions reacted according to Lowry et al. (1951)].

Density gradient centrifugation of I-Bgt-labeled crude mitochondrial fraction membranes in Percoll or sucrose resolves a major peak of toxin binding activity ($\rho < 1.040$ g/mL in Percoll; $\rho \simeq 1.137$ g/mL in sucrose) from less dense material that has little to no toxin binding (Figure 3). In ad-

Table II: Ligand Competition Potency Profile[a]

ligand	K_i^{app} (μM)	ligand	K_i^{app} (μM)
nicotine	0.9	decamethonium	70
acetylcholine	0.9	succinyldicholine	70
bromoacetylcholine	2.0	succinylcholine	70
d-tubocurarine	3.0	atropine	100
tetramethylammonium	3.0	benzoquinonium	130
acetylthiocholine	3.0	methachol	160
carbamylcholine	3.0	choline	200
alcuronium	3.0	dihydro-β-erythroidine	200
hemicholinium	5.0		
phenyltrimethylammonium	13	trimethylammonium	260
butyrylthiocholine	16	muscarine	260
tetraethylammonium	20	β-erythroidine	1000
lobeline	27	hexamethonium	1300
suberyldicholine	27	mecamylamine	1300
pancuronium	50	pilocarpine	2300
gallamine	70	scopolamine	2600
		biperidin	2600

[a] Drugs were tested at a variety of concentrations in preincubation competition assays toward I-Bgt binding. The concentration for ligand that inhibits toxin binding by 50% (IC_{50}) is used to determine the apparent inhibition constant K_i^{app} for ligand binding to toxin sites according to the formula $K_i^{app} = IC_{50}(1 + [\text{I-Bgt}]\bar{K}'^{-1})^{-1}$ where $\bar{K}'$ is the apparent dissociation constant for the high-affinity toxin binding site for the incubation period used.

dition, a minor fraction of I-Bgt binding material is observed on both sucrose (ρ = 1.15 g/mL) and Percoll (broadly dispersed at $\rho > 1.04$ g/mL) gradients. Both major and minor components of the toxin binding site distribution closely follow the profile for membrane protein.

Phase partitioning of brain membranes in poly(ethylene glycol)–dextran results in no net purification of I-Bgt binding sites, although both toxin sites and total protein are concentrated at the phase interface. By comparison, AcChoR-rich membranes from *Torpedo* are enriched at the phase interface, and to a lesser extent in the upper phase, under the same conditions (data not shown).

Approximately 50% of I-Bgt binding sites are solubilized on incubation in Ringer's medium supplemented with 1% cholate, deoxycholate, Triton X-100, or octyl glucoside. When a buffer solution without divalent cations is substituted, about 60% of toxin sites are solubilized. Treatments of I-Bgt-labeled crude mitochondrial fractions with 1 M NaCl or at pH 11.0 are ineffective in solubilizing toxin sites.

Pharmacological Characterization of I-Bgt Binding Sites. I-Bgt binding to crude mitochondrial fraction membranes is

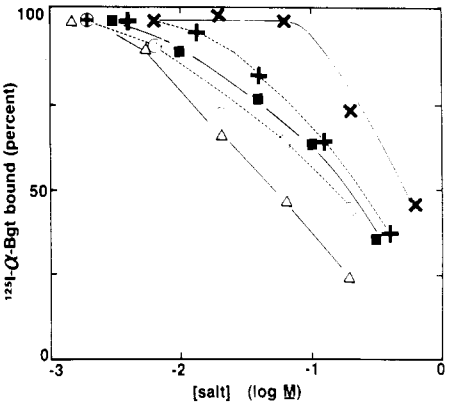

FIGURE 4: Ionic strength dependence. I-Bgt binding (percent maximal) is plotted against buffer or salt concentration for buffers comprised of Ringer's salts with the following modifications: (×) 0–0.6 M NaCl; (+) 0–0.4 M KCl; (○) 0–0.2 M $MgSO_4$; (△) 0–0.2 M $CaCl_2$; (■) 5 mM $NaHCO_3$, pH 7.4 – 0–0.3 M Tris. All resuspensions were done in the appropriate solution without the test substance.

insensitive to variation from pH 4.5 to 9.4. The ionic strength dependence of I-Bgt binding was assessed by varying the concentration of each of the constituents of Ringer's solution. The results (Figure 4) show sensitivity of specific toxin binding to the presence of KCl, divalent cations, and Tris at concentrations greater than 10 mM, and NaCl at 100 mM or higher. Thus, a slight competitive inhibition of toxin binding in full Ringer's solution may be expected due to the presence of Tris, but the other Ringer's salts appear only to diminish nonspecific binding levels.

To test the pharmacological potency of cholinergic ligands acting at I-Bgt binding sites, crude mitochondrial fractions were preincubated with ligands prior to exposure to I-Bgt. The results (Table II) indicate a distinct nicotinic-type pharmacology for the toxin binding site.

Preincubation competition experiments with native Bgt and najatoxin yield inhibition curves that agree well with radiolabeled toxin saturation profiles. Both toxins yield K_i^{app} values of 1–3 nM on coincubation competition. Note that Bgt, najatoxin, and a variety of other fractions from *Bungarus* and *Naja* venoms block 100% of high-affinity I-Bgt binding (data not shown).

As was previously demonstrated for toxin binding sites in Wag/Rig rat brain (Lukas & Bennett, 1979a), toxin binding sites in Simonsen-Albino rat brain crude mitochondrial fraction membranes exhibit unique sensitivity to exposure to nicotinic cholinergic agonists. Toxin binding sites display high affinity

Table III: Acetylcholine Inhibition of I-Bgt Binding following Affinity-State and Disulfide/Sulfhydryl Modification[a]

treatment	K_i^{app} (μM) Ringer's	K_i^{app} (μM) Ca^{2+} free	treatment	K_i^{app} (μM) (Ringer's)
none	2 (9)	5 (15)	DTT-ACh/NEM	90
DTT-DTNB	2 (9)	8 (15)	DTT-d-TC/NEM	90
DTT	11 (90)	270 (270)	DTT-nic/NEM	20
DTT-NEM	60 (120)	>300 (>300)	DTT-najatoxin/NEM	100
ACh/DTT-NEM	2	10	NEM	3
nic/DDT-NEM	3		ACh/NEM	2
carb/DDT-NEM	2		d-TC/NEM	3
d-TC/DTT-NEM	60	200	NEM-DTT	12
alc/DTT-NEM	60		ACh/NEM-DTT	12
			d-TC/NEM-DTT	15

[a] Acetylcholine competition curves were generated as described under Experimental Procedures for inhibition of I-Bgt binding to crude mitochondrial fraction membranes, treated with various cholinergic ligands and/or sulfhydryl/disulfide reagents as indicated. Values of apparent K_i are calculated from the data according to the formula provided in Table II for experiments following preincubation, and coincubation (data in parentheses), paradigms. Abbreviations: DTT, 0.3 mM dithiothreitol; NEM, 0.3 mM *N*-ethylmaleimide; NbS_2, 0.3 mM 5,5'-dithiobis(2-nitrobenzoic acid); ACh, 500 μM acetylcholine; nic, 200 μM nicotine; carb, 500 μM carbamylcholine; d-TC, 500 μM *d*-tubocurarine; alc, 200 μM alcuronium; najatoxin, 100 nM najatoxin.

for agonist when preincubated with agonist prior to exposure to radiotoxin, relative to apparent low affinity for agonist on simultaneous exposure of membranes to small ligand and radiotoxin (Table III). These toxin binding site affinity-state transitions are specific to nicotinic agonists, insofar as they are not observed for reagents such as *d*-tubocurarine, muscarine, or atropine.

The affinity of toxin binding sites for agonist is sensitive to the presence of Ca^{2+} in the incubation medium (Table III). Agonist binding affinity is not modulated, however, by catecholamines, muscarinic cholinergic ligands, cyclic nucleotides, GTP, ATP, anticonvulsant drugs, barbiturates, or benzodiazepines (data not shown).

Affinity of toxin sites for nicotinic agonist is also sensitive to modification of membrane sulfhydryl and disulfide residues. In ligand preincubation experiments (Table III), reaction of membranes with dithiothreitol, known to cleave disulfides and reduce sulfhydryls, results in approximately 5-fold lower affinity of toxin binding sites for acetylcholine. The dithiothreitol effect is fully reversible on treatment of reduced membranes with NbS_2. A low-affinity state of the toxin binding site is also preserved by alkylation of dithiothreitol-reduced membranes with *N*-ethylmaleimide. In the cases of native, reduced and oxidized, reduced, or reduced and alkylated preparations, toxin binding sites display lower affinity for acetylcholine when the coincubation paradigm is used. Moreover, when experiments are performed in Ca^{2+}-free Ringer's medium, both preincubation and coincubation IC_{50} values are increased for all sulfhydryl/disulfide treatments. Coincubation IC_{50} to preincubation IC_{50} ratios are reduced but preserved in Ca^{2+}-free medium for native and for reduced and oxidized membranes but are abolished for reduced and for reduced and alkylated preparations (Table III). All disulfide/sulfhydryl effects and effects of Ca^{2+} on ligand binding affinities are observed only for acetylcholine and carbamylcholine, but not for cholinergic antagonists or muscarinic ligands. There are two other remarkable observations. First, the affinity of toxin binding sites for nicotine is influenced by chemical modification and agonist-induced affinity-state transitions, but only to an extent that is intermediate between effects seen for acetylcholine and for *d*-tubocurarine. Second, toxin binding rate experiments demonstrate an approximate 50% reduction in the observed association rate constant for I-Bgt binding to dithiothreitol–*N*-ethylmaleimide-treated membranes.

When acetylcholine, nicotine, or carbamylcholine, but not *d*-tubocurarine or alcuronium, is incubated with membranes just prior to treatment with dithiothreitol, toxin binding sites maintain high affinity for agonist (Table III). The concentration dependence for this agonist-mediated protective effect is the same as the concentration dependence for inhibition of I-Bgt binding. If ligand or najatoxin is added to membranes already reduced by treatment with dithiothreitol, but before addition of *N*-ethylmaleimide, even to concentrations of acetylcholine or *d*-tubocurarine in excess of 30 mM or of najatoxin at 1 μM, toxin binding sites exhibit the same low affinity for agonist as for dithiothreitol–*N*-ethylmaleimide-treated sites. Membranes treated with *N*-ethylmaleimide, or *N*-ethylmaleimide–dithiothreitol, are no different with respect to ligand competition experiments than native or dithiothreitol-treated membranes, respectively, and the results obtained are not influenced by preincubation with cholinergic ligand.

As first demonstrated with toxin binding sites from Wag/Rig rat brain (Lukas & Bennett, 1980b), the nicotinic receptor affinity reagent bromoacetylcholine alkylates I-Bgt binding sites in Simonsen-Albino rat brain that have been

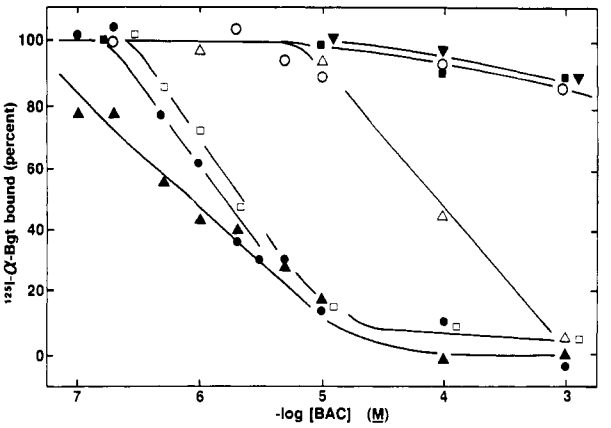

FIGURE 5: Bromoacetylcholine interaction with I-Bgt binding sites. I-Bgt binding (percent maximal) is plotted against the molar concentration of bromoacetylcholine (log scale) used to treat native (○) or dithiothreitol-treated (●) membranes. Other treatments preceding bromoacetylcholine reaction are (▼) 200 μM acetylcholine/dithiothreitol–, (▲) dithiothreitol–200 μM acetylcholine/; (△) dithiothreitol–500 μM *d*-tubocurarine/*N*-ethylmaleimide–; (□) 200 μM acetylcholine/dithiothreitol–*N*-ethylmaleimide–dithiothreitol–; (■) 500 μM *d*-tubocurarine/dithiothreitol–*N*-ethylmaleimide–dithiothreitol–.

Table IV: Affinity Reagent Blockade of I-Bgt Binding[a]

treatment	K_{50} (μM)	treatment	K_{50} (μM)
none	>1000	DTT-Bgt/	>1000
ACh/	>1000	DTT-najatoxin/	>1000
d-TC/	>1000	DTT-NEM-DTT-	>1000
DTT-	2	ACh/DTT-NEM-DTT-	2
ACh/DTT-	>1000	d-TC/DTT-NEM-DTT-	>1000
d-TC/DTT-	4	DTT-ACh/NEM-DTT-	>1000
DTT-ACh/	0.8	DTT-d-TC/NEM-DTT-	100
DTT-carb/	1	DTT-najatoxin/NEM-DTT-	5
DTT-nico/	2	NEM-DTT-	2
DTT-d-TC/	100	ACh/NEM-DTT-	2
DTT-alc/	300	d-TC/NEM-DTT-	2

[a] Aliquots of membrane fractions, treated as specified below, are reacted with bromoacetylcholine at concentrations from 0 to 1 mM. Following removal of unbound reagent, I-Bgt binding assays in Ringer's buffer at 10 nM radiotoxin are performed. K_{50} values are the concentration of bromoacetylcholine necessary to block 50% of toxin binding. All abbreviations are as in Table III, with Bgt signifying incubation at 10 nM toxin.

reduced by treatment with dithiothreitol and irreversibly blocks toxin binding (Figure 5; Table IV). Alkylation of reduced sufhydryls with *N*-ethylmaleimide prior to rereduction and exposure to bromoacetylcholine abolishes irreversible inhibition of toxin binding. If membranes are preincubated with agonist, but not antagonist, prior to reduction with dithiothreitol and *N*-ethylmaleimide-mediated alkylation, their ability to subsequently be reduced and affinity labeled with bromoacetylcholine is preserved. Similarly, preincubation of membranes with agonist, but not antagonist, prior to treatment with dithiothreitol protects against affinity alkylation by bromoacetylcholine. When acetylcholine at concentrations as high as 30 mM is incubated with membranes that already have been treated with dithiothreitol, subsequent reaction with *N*-ethylmaleimide is apparently unhindered, as bromoacetylcholine reactivity cannot be regenerated. In contrast, native najatoxin at 100 nM or *d*-tubocurarine at 1 mM protects the reduced bromoacetylcholine site from alkylation with *N*-ethylmaleimide. When reduced membranes are exposed to nicotinic agonists just prior to addition of bromoacetylcholine, affinity alkylation is enhanced slightly relative to experiments where no agonist is added. Again, by contrast, *d*-tubocurarine

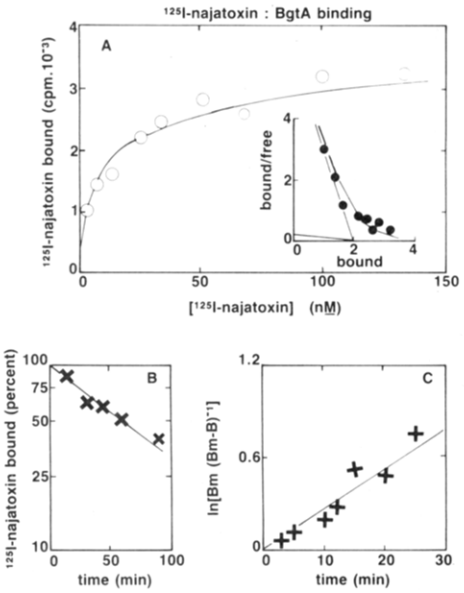

FIGURE 6: I-Najatoxin binding. (A) Specific binding of I-najatoxin to crude mitochondrial fractions (cpm × 10^{-3}) is plotted in linear fashion against radiotoxin concentration (nM). The insert shows a modified Hofstee–Eadie–Scatchard representation of the data. Solid lines are theoretical curves according to the equation described in the legend to Figure 1 for $V'_1 = V'_2 = 2000$ cpm, $\bar{K}'_1 = 3$ nM, and $\bar{K}'_2 = 100$ nM. (B) Dissociation of I-najatoxin is plotted as radiotoxin bound (%) on a log scale against time of physical dilution. (C) Association of I-najatoxin is plotted as described in the legend to Figure 2.

or alcuronium at 500 μM partially blocks and Bgt at 10 nM (or najatoxin at 100 nM) completely blocks affinity alkylation or reduced membranes. Affinity alkylation is not achieved by incubation of agonist or antagonist with membranes prior to exposure to bromoacetylcholine.

Characterization of I-Najatoxin Toxin Binding Sites. Preequilibrium binding isotherms and dissociation and association kinetic curves for interaction of I-najatoxin with specific binding sites in Simonsen-Albino rat brain are illustrated in Figure 6. The saturation isotherm and its transformation according to Hofstee–Eadie–Scatchard indicate the presence of two classes of binding site, with $\bar{K}'$ values of 3 and 100 nM, in an approximate 1:1 stoichiometry. The dissociation half-life of about 60 min yields a value for k_{-1} of 0.0116 min^{-1}. The association rate, k_1', is 0.027 min^{-1} at 10 nM I-najatoxin, yielding $k_1 = 0.0027$ min^{-1} nM^{-1}.

Preincubation competition experiments toward I-najatoxin binding were used to generate a pharmacological profile for toxin sites. Native Bgt and najatoxin are effective inhibitors of I-najatoxin binding at nanomolar concentrations. The rank order of inhibition potency of cholinergic ligands follows closely the pharmacological profile for inhibition of I-Bgt binding (Table V). Modification of I-najatoxin sites with sulfhydryl and disulfide reagents modulates affinity of those sites for acetylcholine (Table V). Reaction of dithiothreitol-reduced toxin sites with bromoacetylcholine irreversibly blocks radiotoxin binding.

Table V: Pharmacological Profile for I-Najatoxin Binding Site[a]

Preincubation Competition			
ligand	K_i^{app} (μM)	ligand	K_i^{app} (μM)
nicotine	0.7	lobeline	7
acetylcholine	1	gallamine	70
d-tubocurarine	4	pancuronium	90
bromoacetylcholine	6	decamethonium	90
carbamylcholine	6	atropine	90
alcuronium	6	hexamethonium	>1000

Sulfhydryl/Disulfide Modification; Acetylcholine Competition			
treatment	K_i^{app} (μM)	treatment	K_i^{app} (μM)
none	1	DTT	10
DTT-DTNB	1	DTT-NEM	100

Bromoacetylcholine Reaction	
treatment	K_{50} (μM)
none	>1000
DTT	10
DTT-NEM-DTT	>1000

[a] Toxin binding inhibition studies (preincubation paradigm), sulfhydryl/disulfide group modification, and bromoacetylcholine affinity alkylation are carried out as described for I-Bgt studies in Ringer's buffer. Abbreviations used are as defined in Tables II–IV.

Discussion

Monoiodinated Bgt, labeled with ^{125}I, and purified at 4 °C, pH 6.0, by gradient elution on a carboxymethylcellulose column, has been used as a ligand to label putative nicotinic acetylcholine receptors in the central nervous system of the Simonsen-Albino rat. Some of the data reported here confirms and extends results of our previous investigations with Wag/Rig rats. A brief discussion of the confirmatory results follows. More detailed discussion of the major points highlighted here is given in previous publications [see Lukasiewicz & Bennett (1978), Lukas & Bennett (1979a,b, 1980a,b), and Lukas et al. (1979)].

The present data show high-affinity, specific binding of I-Bgt to crude mitochondrial fraction membranes, characterized by a descriptive preequilibrium dissociation constant of approximately 1 nM. ^{125}I-Labeled Bgt dissociates slowly from membrane binding sites with a half-time of about 27.5 h at 21 °C. Association of I-Bgt with toxin binding sites is slower than interactions that are diffusion controlled. Assuming that formation of collision complexes between toxin and binding site is diffusion controlled, it may be inferred that formation of slowly reversible toxin–toxin binding site complexes is at least a two-step process.[2] The physical properties of toxin binding sites are consistent with their localization in subcellular fractions containing synaptic plasma membranes and/or junctional complexes. Morphological heterogeneity of CNS synapses and the possible extrasynaptic disposition of toxin binding sites encumber efforts toward further enrichment of sites on subcellular fractionation. The pharmacological profile of drugs competing for high-affinity, specific radiotoxin binding in physiological solution is distinctly nicotinic cholinergic in character. The affinity of I-Bgt binding sites for nicotinic cholinergic agonists is uniquely sensitive to preincubation with agonist, modification of binding site disulfides/sulfhydryls, and the presence of Ca^{2+}. Moreover, the nicotinic receptor affinity reagent bromoacetylcholine interacts with dithiothreitol-reduced toxin binding sites and irreversibly blocks I-Bgt binding. Taken together with results from other laboratories [reviewed by Schmidt et al. (1980) and Morley & Kemp (1981)], these results suggest considerable physical and chemical homology between mammalian CNS Bgt binding sites and authentic AcChoR from the periphery. Evidence outlined above also suggests that high- ($\bar{K}'_1 \simeq 1$ nM) and low- ($\bar{K}'_2 \simeq 100$ nM) affinity I-Bgt binding sites exist on rat crude mitochondrial fraction membranes. More detailed studies of low-affinity I-Bgt binding sites, and a discussion of their potential physiological relevance, are presented elsewhere (Lukas, 1984).

The result indicating that 2–4-fold purification of Bgt binding sites may be achieved by following the synaptic

junctional complex purification scheme developed by Cotman & Taylor (1972) and Davis & Bloom (1973) provides further support for localization of toxin sites at CNS synapses. While putative receptors for aspartate and glutamate are purified approximately 9-fold by a similar procedure (Foster et al., 1981), the present data may reflect greater morphological heterogeneity of synaptic and extrasynaptic Bgt binding sites and/or higher solubility of junctional toxin binding sites in Triton X-100 and other detergents.

Toward further characterization of CNS Bgt binding sites, other new data presented above indicate that bromoacetylcholine fails to label Bgt sites when membranes are preincubated with agonist prior to reduction with dithiothreitol. This result suggests that irreversible labeling of toxin sites with [^{3}H]bromoacetylcholine may be achieved with favorable signal to noise ratios by first performing reduction and alkylation of nontoxin binding site sulfhydryls with nonradiolabeled alkylating agent in the presence of agonist.

Once toxin binding sites are reduced with dithiothreitol, agonist (1–500 μM acetylcholine) actually enhances affinity labeling with bromoacetylcholine. On the other hand, the most potent inhibitors of bromoacetylcholine interaction with dithiothreitol-reduced toxin binding sites are Bgt and najatoxin (10–100 nM), followed by alcuronium and *d*-tubocurarine (approximately 1 mM), while other agonists are ineffective even at concentrations to 100 mM. The same rank order of potency is also observed when toxins and drugs are tested for their ability to protect the reduced bromoacetylcholine interaction site from nonspecific alkylation with *N*-ethylmaleimide. From these data, it is suggested that affinity alkylation sites (i.e., the class of reduced sulfhydryls that interact with bromoacetylcholine) are located proximal to the cholinergic ligand binding site and that large ligands sterically inhibit specific, bromoactetylcholine-mediated and nonspecific, *N*-ethylmaleimide-mediated alkylation of the proximal, affinity alkylation sulfhydryls. It is further suggested that agonist-induced desensitization of toxin binding sites prevents exposure of proximal, affinity alkylation sulfhydryls/disulfides to dithiothreitol but that agonist incubation with reduced membranes leaves toxin binding sites in yet another unique state, with enhanced accessibility of bromoacetylcholine to reduced, proximal, affinity alkylation sulfhydryls.

In contrast to their effects on alkylation of proximal sulfhydryls, toxins, antagonists, and agonists are all ineffective in preventing alkylation (and consequent shift of toxin binding sites to a low-affinity state toward agonist) of another class of reduced toxin binding sites with *N*-ethylmaleimide [see above and Lukas & Bennett (1980a)]. Thus, while the reduction–alkylation sequence does result in slower Bgt binding, it is likely that at least part of this class of sulfhydryls is located distal to the acetylcholine binding site. Nevertheless, agonist-specific protection against reduction–alkylation of distal and proximal sulfhydryls/disulfides is equally effective, suggesting global burial of toxin binding site disulfides/sulfhydryls on exposure to agonist.

New information from this laboratory on binding of I-najatoxin is in substantial agreement with an earlier report (Speth et al., 1977). The binding sites for radiolabeled Bgt and najatoxin appear to be the same on the basis of mutual competition potency, comparable pharmacological profiles, and sensitivity to sulfhydryl/disulfide modification and affinity alkylating reagent. The binding of both radiolabeled toxins to brain sites is characterized by presence of high- and low-affinity components.

Complete characterization of CNS curaremimetic neurotoxin binding sites shall require development of more highly purified and less heterogeneous preparations of toxin binding sites, in both membrane-bound and solubilized states, than the preparations described above. Nevertheless, the sensitivity and specificity of neurotoxin binding assays have permitted delineation of fundamental properties of toxin binding sites and provided a foundation for more detailed biochemical studies. Definitive evidence that toxin binding sites are biologically relevant receptors will rest on physiological and pharmacological studies of toxin action.

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Supplementary Material Available

Chromatographic profiles of venom fractionation, toxin purification, and radiolabeled toxin preparation, isolation, and characterization, primary data concerning agonist-mediated, sulfhydryl/disulfide group modification mediated, and Ca^{2+}-mediated effects on ligand competition toward toxin binding, and ligand-induced alterations in toxin binding site sulfhydryl/disulfide reactivity (6 pages). Ordering information is given on any current masthead page.

Registry No. α-Bungarotoxin, 11032-79-4.

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Detection of Low-Affinity α-Bungarotoxin Binding Sites in the Rat Central Nervous System†

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ABSTRACT: The curaremimetic neurotoxin, α-bungarotoxin, is shown to interact with two classes of binding sites on rat brain crude mitochondrial fraction membranes. Toxin binding sites are characterized by descriptive, preequilibrium dissociation constants of about 5 and 400 nM. There are at least as many low-affinity toxin binding sites as high-affinity sites. The existence of low- and high-affinity sites is confirmed by experiments with native toxin. Low- and high-affinity toxin binding sites are copurified as judged by sedimentation velocity and density gradient analysis, consistent with the presence of low- and high-affinity toxin binding sites on the same subcellular membrane fragments. The results may offer an explanation for the relatively low antagonistic potency of curaremimetic neurotoxins at acetylcholine-sensitive sites in the vertebrate central nervous system.

Use of curaremimetic neurotoxins as molecular probes in characterization of nicotinic acetylcholine receptors (nAcChoR)[1] in the periphery (Conti-Tronconi & Raftery, 1982) is founded on demonstration of their potent antagonistic action (at submicromolar concentrations) at the vertebrate neuromuscular junction and on the electric organ of ray and eel (Lester, 1970; Lee, 1972). Application of neurotoxins as probes for autonomic and central nervous system nAcChoR (Moore & Loy, 1972; Greene et al., 1973; Salvaterra & Moore, 1973; Simantov & Sachs, 1973; Eterovic & Bennett, 1974; Romine et al., 1974), however, predated rigorous tests of their physiological potency in the autonomic nervous system (ANS) or central nervous system [CNS; see Morley & Kemp (1981) but see also Chou & Lee (1969)]. A review of the literature indicates that curaremimetic neurotoxin antagonistic potency at some loci in the ANS and CNS is reduced, but finite and significant (see Discussion). That is, only at 1–10 μM α-bungarotoxin (Bgt) is the response to acetylcholine (AcCho) or stimulation of cholinoceptive elements blocked (Fex & Adams, 1978; Dun & Karczmar, 1980; Marshall, 1981; Syapin et al., 1982).

Due to technical difficulties and limitations in signal to noise ratios, studies of ANS and CNS Bgt binding have focused on high-affinity sites with apparent dissociation constants on the order of 1 nM. A number of studies have shown that occupation of these sites is inadequate to block putative cholinoceptive responses in the mammalian CNS or ANS (see Discussion). Any attempt to resolve this apparent discrepancy in toxin antagonistic potency and binding site specificity requires coincident study of Bgt binding to sites with relatively low affinity for toxin. For example, if there exist toxin binding sites with an apparent dissociation constant on the order of 100 nM, one would predict occupation of 91 and 99% of those sites at toxin concentrations of 1 and 10 μM, respectively, which may provide detectable expression of toxin-mediated antagonism.

Using freshly prepared ^{125}I-labeled monoiodinated α-bungarotoxin (I-Bgt), and a centrifugation assay with Simonsen-Albino rat brain crude mitochondrial fractions, we have detected a class of Bgt binding sites with relatively low affinity for toxin [see Lukas (1984)]. Data are presented in this paper that further characterize this class of toxin binding sites.

Experimental Procedures

Purification and iodination of Bgt, characterization of radiolabeled toxin, and preparation of rat brain crude mitochondrial fraction membranes are described elsewhere (Lukas, 1984). The general outline of I-Bgt binding and kinetics assays, density gradient centrifugation and sedimentation velocity experiments, affinity-labeling protocols, and preincubation and coincubation ligand competition experiments are also according to procedures detailed in other papers (Lukas

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[1] Abbreviations: nAcChoR, nicotinic acetylcholine receptor; ANS, autonomic nervous system; CNS, central nervous system; Bgt, α-bungarotoxin; AcCho, acetylcholine; I-Bgt, ^{125}I-labeled monoiodinated α-bungarotoxin; d-TC, *d*-tubocurarine.

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