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α -BUNGAROTOXIN BINDING PROPERTIES OF A CENTRAL NERVOUS SYSTEM
NICOTINIC ACETYLCHOLINE RECEPTOR

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SUMMARY

High-affinity, specific binding of radiolabeled α -bungarotoxin (α -Bgt) to particulate fractions derived from rat brain shows saturability ($B_{\max} \approx 37$ fmole/mg, $K_D^{\text{app}} = 1.7$ nM), insensitivity to ionic strength, and is essentially irreversible ($K_{\text{on}} = 5 \cdot 10^6$ /min mole; $K_{\text{displacement}} = 1.9 \cdot 10^{-4}$ /min, $\tau_{1/2} = 62$ hrs). Subcellular distribution of specific sites is consistent with their location on synaptic junctional complex and post-synaptic membranes. These membrane-bound binding sites exhibit unique sensitivity to cholinergic ligands; pretreatment of membranes with cholinergic agonists (but not antagonists) induces transformation of α -Bgt binding sites to a high affinity form toward agonist. The effect is most marked for the natural agonist, acetylcholine. These results strongly support the notion that the entity under study is an authentic nicotinic acetylcholine receptor.

INTRODUCTION

Neurotransmitter receptors are defined as those physical entities that, on response to exposure to neurotransmitter substances released from the pre-synaptic neuron, initiate cation translocation across the post-synaptic effector-cell membrane, triggering post-synaptic depolarization. By their nature, neurotransmitter receptors should possess high-affinity binding sites for natural transmitter substances. They should also be coupled to an ion channel or some other entity responsible for maintenance of the membrane potential, and this coupling should be sensitive to the presence of neurotransmitter. Furthermore, the dynamics of this coupling phenomena and/or the primary interaction between receptor and neurotransmitter itself should differ in response to agonistic as opposed to antagonistic influence.

In order to identify the fundamental physical determinants of these receptor functions, and to determine which properties are unique to a particular receptor, or are universal to all receptors, biochemical characterization of both peripheral and central nervous system receptors is necessary. Since the original biochemical description of the properties of post-synaptic receptors in the central nervous system [1], most attention has turned to cholinergic receptors found at the vertebrate neuromuscular junction and in electric organ, and an extensive literature on biochemical and physiological characterizations of peripheral nicotinic acetylcholine receptors (nAChR) has [2] and continues to develop. Concurrently, with the utilization of radiolabeled stereospecific and natural ligands [3-5], putative central nervous systems (CNS) neurotransmitter receptor entities began to be characterized. Toward identification of a CNS nAChR, reports from this [6] and other laboratories [7-13] have appeared describing the specificity of krait α -toxin binding to particulate and detergent-solubilized fractions derived from mammalian brain. However,

other than the ability of cholinergic drugs to prevent α -toxin binding, which might be expected on the basis of their comparable structural features alone, there has appeared no demonstration of active responsiveness of the α -toxin binding entity derived from mammalian brain to cholinergic ligands.

Reported here are the characteristics of a membrane-bound entity from rat brain capable of high-affinity, specific interaction with the post-synaptic toxin from Bungarus multicinctus, α -bungarotoxin (α -Bgt). Insensitivity of this interaction to non-specific ionic strength effects is described, as well as a partial characterization of alteration in receptor binding properties on exposure to cholinergic ligands, and selectivity of that response to agonists. A summary report of these results was presented at the annual meeting of the American Society for Neurochemistry, Washington, D.C., March, 1978 [14].

METHODS

Procedures for purification of α -Bgt from crude lyophilized venom of Bungarus multicinctus (Miami Serpentarium) and preparation and properties of ^{125}I -labeled α -toxin ($[^{125}\text{I}]_1 \alpha$ -Bgt) and tritiated α -Bgt ($[^3\text{H}] \alpha$ -Bgt) are as described previously [6,15-16].

Preparation of Brain Membrane Fractions

Wag/Rig rats (Lawrence Berkeley Laboratory rat colony) are decapitated and ice-chilled sub-cortices and cortices (unless otherwise noted, hindbrain, and cerebellum are discarded) are homogenized in 10 volumes of 0.32M sucrose pH 7.0, 0°C, with 10 up and down motions of a teflon pestle driven at 1000 rpm by a Mixmaster (Sunbeam) within a pyrex homogenizer. In some experiments, 0.5mM NaH_2PO_4 pH 7.5, 10 μ M phenylmethyl sulfonyl fluoride (PMSF) and 0.01% NaN_3 were included in the isotonic sucrose solution without altering experimental results. The homogenate is centrifuged at 1000g

(2500 rpm, Sorvall RC-3 centrifuge, 4°C) for 10 min and supernatants collected, taking care to reject the white fluff layered just above the crude nuclear fraction pellet. The pellet is resuspended in 2 volumes of homogenizing solution and recentrifuged, supernatants are pooled as above and subjected to sedimentation at 17 800g (12 000rpm, Sorval RC-2B, SS-24 rotor, 4°C) for 15 min (unless otherwise noted) to yield crude mitochondrial fractions in the pellet, and microsomal fractions as supernatants. Routinely, mitochondrial fractions are then resuspended in binding Ringers (115mM NaCl, 5mM KCl, 1.8mM CaCl₂, 1.3mM MgSO₄, 33mM Tris, pH 7.4 with HCl), sedimented at 19 000g for 10 min, before resuspension again in Ringers for binding assays as crude mitochondrial fraction membranes (CMF). When so indicated, mitochondrial fractions are subjected to osmotic shock in 2mM Tris, pH 7.4, and resuspended in appropriate solution for assays.

Binding Assays

Levels of α -Bgt binding are usually determined by incubating brain particulate fractions with radiolabeled α -Bgt for 60 min in a volume of 250-750 μ l at 21°C with shaking. Upon termination of the assay, the suspension is diluted in 3 ml of wash solution and sedimented at 18 000g for 10 min (RC-2B, SS-24 rotor, 4°C) and the pellet is resuspended in 3 ml of wash solution. The sequence of sedimentation and resuspension, a wash cycle, is repeated one to three times. Upon completion of the wash routine, resuspended pellets are quantitatively transferred either to vials containing Aquasol 2 (Packard) for determination of ³H by liquid scintillation counting (25-40% efficiency, Packard Tricarb 3375) or to γ -well counting tubes for determination of ¹²⁵I by γ -ray spectroscopy (41% efficiency, Nuclear Chicago). Two distinct assay procedures are used for determination of specific binding levels. Non-specific binding is determined by pretreating particulate fractions with an excess of

native α -Bgt for at least 30 min prior to exposure to radiolabeled α -toxin. Under these conditions, no specific α -Bgt binding sites are occupied by radiolabeled toxin, and radiolabeled toxin binding to any non-specific sites is diluted in proportion to the ratio of native to radiolabeled toxin. Total binding levels are determined in samples which are chased with excess native toxin for 30 minutes subsequent to treatment with radiolabeled toxin. Under these conditions, all specific sites are complexed with radiolabeled toxin, and the cold chase period serves to dilute radiolabeled toxin binding to non-specific sites to the same extent as in the non-specific binding determination procedure. Thus, specific binding levels, i.e., total minus non-specific binding, accurately reflect the number of specific α -Bgt binding sites. It should be made explicit that this routine is based on assumption that the specific interaction of toxin and receptor is long-lived, while non-specific interactions reach equilibrium within one-half hour and decay rapidly. The extensive wash routine is also designed on that premise. Whether the wash solution is composed of binding Ringers, a phosphate-buffered NaCl solution (5mM Na_2HPO_4 , pH 7.4, 100mM NaCl) or low ionic strength Tris buffer (2mM, pH 7.4) specific binding levels are unaltered through at least 5 wash cycles over four hr, while an increased-number of washes or an increase in the ionic strength of the wash solution act to decrease non-specific binding only. For determination of rate constants for toxin-membrane complex formation, the period of incubation with radiolabeled toxin is varied. For determination of rate of complex decay the cold chase period is extended as indicated. In drug competition studies, CMF is preincubated with the appropriate ligand for the period indicated prior to exposure to radiolabeled toxin, or the reaction is initiated by addition of CMF to ligand and toxin in solution for coincubation studies. In these experiments, the cold chase serves to terminate the specific

interaction of radiolabeled toxin with CMF, as well as equilibrate non-specific binding levels. Details concerning the concentrations of reagents and composition of the assay solution are indicated in Figures and Legends.

Solubilization Experiments

For determination of the relative effectiveness of non-ionic detergents toward solubilizing α -Bgt binding sites, multiple, identical samples of CMF are processed for determination of total and non-specific α -Bgt binding as described above except that final, washed pellets are then resuspended in the appropriate solvent. After 1 hour incubation at 20°, insoluble material is sedimented at 39 000g for 30 min. Precipitable total and non-specifically bound radioactivity is determined by filtration of ammonium-sulfate treated samples on Millipore HAWP filter discs [17] or by the Whatman DE81 filter disc method [18] with comparable results. Non-specific adsorption of radiolabeled toxin to filters is negligible given the small quantities of free toxin in these experiments. In all cases, material balance of bound radioactivity is within 10% of control values for CMF treated in the absence of detergent. Assays for α -Bgt binding to Triton X-100 solubilized CMF is as described above, except that filter disc methods [17,18] are substituted for wash procedures. For these assays, the cold chase protocol is particularly useful to minimize non-specific adsorption of radiolabeled toxin to filter discs. The most consistent results were obtained using a single disc per assay rather than a stack of two discs; criteria for accuracy and consistency of filtration assay results is the extent of agreement with results obtained on duplicate samples using more tedious gel filtration or ion-exchange column chromatography techniques.

Other Methods

Membrane proteins were determined by conventional, and solubilized proteins by modified Lowry [19] techniques, using protein standards made in the presence of detergent.

Acetylcholine perchlorate was synthesized in our laboratory. Other chemicals and reagents were Tris, PMFS, NaN_3 , Triton X-100, carbamylcholine chloride (Sigma); d-tubocurarine chloride, eserine (Calbiochem); nicotine hydrochloride, atropine (K and K Laboratories); hexamethonium chloride dihydrate and sodium deoxycholate (Mann); Emulphogene BC-720 (GAF Corporation); Tween 20 and Tween 80 (Fisher).

RESULTS

Specificity of the interaction of $[^3\text{H}]\alpha\text{-Bgt}$ with membrane preparations derived from crude mitochondrial fractions is demonstrated by the results of a typical binding saturation experiment shown in Fig. 1. Non-specific binding increases linearly with the concentration of radiolabeled toxin, and amounts to about 40% of total binding levels at 10nM $[^3\text{H}]\alpha\text{-Bgt}$.

Saturability of specific $[^3\text{H}]\alpha\text{-Bgt}$ binding sites as determined from the results shown in Fig. 1 and from another experiment using different $[^3\text{H}]\alpha\text{-Bgt}$ and CMF preparations, is illustrated in Fig. 2. Specific $[^3\text{H}]\alpha\text{-toxin}$ binding increases hyperbolically with increasing toxin concentration. The insert in Fig. 2 shows a double reciprocal plot of the binding data for toxin concentrations from 0.5 to 12nM . Least squares linear regression analysis of the data yield a K_D^{app} value of 1.8nM , and maximum specific activity of 38.5 fmole/mg CMF protein. The double reciprocal plot shows some upward curvature (not shown) when specific binding values for $\alpha\text{-toxin}$ concentrations less than 0.6nM are plotted. Table I summarizes the results of numerous saturation experiments for binding of both tritiated and mono-iodinated radiolabeled $\alpha\text{-Bgt}$ derivatives to rat brain CMF. On

the whole, both [$^{125}\text{I}_1$] α -Bgt and [^3H] α -Bgt display the same high affinity for specific CMF sites, and show similar levels for maximum specific binding.

An additional feature of the binding of α -Bgt to mitochondrial fraction membranes is illustrated when the data from Fig. 2 are represented in the form of a Hill plot (Fig. 3). The data may be fit to a single linear regression line (dashed), yielding a slope of 1.39. It is interesting to note that when linear regression analysis is applied to the data for α -Bgt concentrations in the range from 0.6-12nM and from 0 to 0.6nM, the corresponding Hill numbers of 1.16 and 1.93, respectively, approach the significant values of $n = 1.0$ for non-interacting sites $n = 2.0$ for two positively cooperative interacting sites.

An experiment designed to determine the rate of formation of CMF- $[^{125}\text{I}_1]\alpha$ -Bgt complexes is illustrated in Fig. 4, where the variation of total and non-specific α -Bgt binding as a function of time exposed to 10nM toxin is plotted. While non-specific levels of toxin binding are essentially unaffected by the duration of exposure to radiolabeled α -toxin, specific binding increases exponentially with time, saturation being achieved after about 30 min of incubation with [$^{125}\text{I}_1$] α -Bgt. The rate constant for formation of toxin-CMF complexes, is $4.97 \cdot 10^6/\text{min} \cdot \text{mole}$ ($\tau = 14.1$ min). The results of a replicate experiment yield values for the bimolecular process of $6.16 \cdot 10^6/\text{min} \cdot \text{mole}$ for k_1 and $\tau = 11.3$ min. Data demonstrating the irreversibility of CMF- α -Bgt complexes formed after one hour incubation in the presence of 10nM toxin are illustrated in the insert of Fig. 4. Displacement of bound radioactive α -Bgt by an excess of native toxin is fit by a single exponential, with a rate constant of $1.87 \cdot 10^{-4}/\text{min}$; the complex decays with a half-life of 62.3 hours in monophasic fashion.

The effects of ionic strength and buffer concentration on the interaction of α -Bgt with CMF are shown in Fig. 5. As shown in the lower panel, concentrations of Tris buffer above 30mM are found to progressively inhibit the formation of toxin-membrane complexes. Therefore, 2mM Tris buffer was used as a buffering agent in the subsequent experiments and the use of Tris as buffering agent in the Ringers solution used in the bulk of the studies reported here should not adversely affect specific α -Bgt binding. Further, the data show that while levels of non-specific interaction between radiolabeled α -Bgt and brain membranes increase markedly with decreasing concentrations of both divalent (upper panel) and monovalent (lower panel) cations, there is no significant effect on specific binding levels. Thus, increased ionic strength and the concentrations of Ringers salts used in these studies have the effect of decreasing only non-specific binding, hence increasing the ratio of specific to total binding.

Preliminary experiments designed to identify the subcellular distribution of α -Bgt binding sites are summarized in Table II. Fractionation of brain homogenates into operationally defined nuclear (1000g pellets), mitochondrial (1000-9000g pellet) and microsomal (9000-100 000g pellet) fractions resulted in a distribution where the relative activity of specific α -Bgt binding sites was enriched in the microsomal fraction. However, by increasing the centrifugal force under which "mitochondrial" fractions are sedimented, enrichment of toxin binding sites in the "mitochondrial" fraction, at the expense of relative activity in the "microsomal" fraction, is effected. This binding site enrichment routine was, therefore, employed throughout these studies, where the 1000-17 800g "mitochondrial" fraction is used exclusively.

The experiments outlined above have demonstrated saturability and specificity of a high-affinity interaction of α -Bgt with membrane fractions derived from brain, the "irreversible" nature of binding, the resistance of the specific interaction to ionic strength effects, and a subcellular fraction distribution of α -Bgt binding sites in reasonable agreement with that expected for a post-synaptic neurotransmitter receptor molecule. Nevertheless, unequivocal identification of the sites for interaction with α -Bgt as a CNS nAChR rather than another α -Bgt binding protein requires that the cholinergic nature of the binding entity be established. The results of an experiment where cholinergic antagonists and agonists at multiple concentrations are simultaneously incubated with radiolabeled α -Bgt at fixed concentrations in the presence of brain CMF are shown in the upper panel of Fig. 6. Cold native α -Bgt is the most potent competitor for [$^{125}\text{I}_1$] α -Bgt binding sites at equilibrium; nicotine and d-tubocurarine are the most potent of the reversible agonists and antagonists, respectively. Carbamyl choline and acetylcholine (in the presence of 10^{-4}M eserine) are effective only at ca. 10^{-4}M while a ganglionic antagonist, hexamethonium, and a muscarinic antagonist, atropine do not prevent α -Bgt binding at 10^{-3}M . The apparent IC_{50} values from the coincubation condition shown in Fig. 6 are summarized in Table III. While the most potent nicotinic agonists in vivo, namely, acetylcholine and carbamyl choline, are relatively ineffective toward blocking α -Bgt sites on coincubation, their competition effectiveness is markedly increased when preincubated with CMF prior to addition of radiolabeled α -toxin (lower panel, Fig. 6). Nicotine shows a smaller increase in competition effectiveness on preincubation. In contrast, the cholinergic antagonist, d-tubocurarine, is equally potent whether coincubated with toxin or preincubated with membranes prior to

treatment with toxin. There is a slight increase in the effectiveness of native α -Bgt on preincubation, presumably attributable to the "irreversible" nature of its interaction with membrane sites, and atropine and hexamethonium remain impotent at 10^{-3} M. The apparent IC_{50} values for the preincubation condition as well as the ratio of IC_{50} on coincubation to that for preincubation are summarized in Table III. While preincubation with antagonist has little or no effect on the formation of CMF membrane- α -Bgt complexes at equilibrium, pretreatment with agonist results in an apparent increase in the affinity of CMF membrane for agonist. Moreover, this potentiation effect is most evident for the natural ligand, acetylcholine, which apparently binds with forty-fold more tenacity on preincubation than on coincubation; the increase in its apparent affinity for CMF membrane sites is nearly an order of magnitude larger than that seen for artificial cholinergic agonists.

In view of the interest in the ultimate purification of the α -Bgt binding entity characterized above, preliminary experiments were performed using non-ionic detergents to solubilize specific α -Bgt-CMF complexes. As summarized in Table IV, the detergents used showed wide variability in their effectiveness at solubilizing membrane protein and toxin-membrane complexes. With the exception of Emulphogene (32% enrichment) and deoxycholate (95% enrichment), none of the detergents tested in our hands lead to an enrichment of solubilized α -Bgt-CMF complexes. When Triton X-100 solubilized CMF membranes are treated with [125 I] α -Bgt at various concentrations, a typical specific binding saturation curve (here shown for two different experiments, Fig. 7) shows an apparent multiplicity of binding sites, suggesting that additional α -Bgt sites are exposed on solubilization, and/or that the characteristics of the membrane bound α -Bgt binding entity are altered on solubilization.

DISCUSSION

The results presented herein demonstrate the existence of a specific α -Bgt binding component in the rat central nervous system in pmol per gram tissue quantities. The apparent dissociation constant for brain membrane- α -Bgt complex formation, as determined from binding saturation studies, of 1-2 nM, is intermediate to values reported in the literature. Schmidt finds a ten-fold lower K_D^{app} of 0.1 nM [12], while values for K_D^{app} from binding isotherm data of Eterovic and Bennett (6nM, ref. 6), Moore and Brady (8nM, ref 9) and McQuarrie et al (3-10nM, ref 7) are higher. Some of the discrepancies in the results might be explained by the use of di-iodinated α -Bgt [9] as a labeling agent, since it binds to brain membrane fractions with about 1/8 of the affinity of [H^3] α -Bgt or mono- ^{125}I -labeled toxin [15]. In one of the reports [6], no control for non-specific binding levels was included, and the apparent K_D in another report was found to vary as a function of membrane fraction concentration, approaching 1nM when K_D^{app} values are extrapolated to zero protein concentration [7]. In our hands, there has accumulated no evidence for heterogeneity of specific sites, at least with K_D^{app} values differing by an order of magnitude. Rather, there is some evidence that α -Bgt sites display positive cooperativity, or, more likely, that there are two sites per binding entity with similar, if not identical, affinity constants (Hill number of 1.3, upward curvature of double reciprocal plot, ref. 20; see also ref. 15).

The high-affinity, specific interaction of α -Bgt with a CNS entity described here is relatively insensitive to variation in salt concentration, in contrast to the observed behavior of non-specific interactions. Thus, the high ionic strength binding conditions utilized set stringent criteria for specific binding, and demonstrate that toxin binding occurs with high

specificity under assay conditions that approximate those found extracellularly, i.e., conditions that presumably prevail in the vicinity of extracellular membrane recognition sites. This finding is in contrast to other reports where 200mM NaCl is used to terminate or prevent "specific" α -Bgt binding to whole brain homogenates [12] and where marked ionic-strength sensitivity of α -Bgt binding to brain crude mitochondrial fractions is described [7]. In the latter report, the variation of binding with ionic strength closely resembles the variation of total (non-specific plus specific) binding reported here.

The essentially "irreversible" nature of CMF- α -Bgt complex formation is illustrated by a displacement rate of $3.1 \cdot 10^{-6}$ /sec ($\tau = 62$ hrs), comparing favorably with that found for identified nAChR from electric organ [21] under similar assay conditions, while being slower than that reported for brain membrane α -Bgt binding ($5.8 \cdot 10^{-5}$ /sec, $\tau = 3.5$ hr; ref 12) and toxin binding to solubilized brain particulate fractions ($1.2 \cdot 10^{-5}$ /sec, $\tau = 15.6$ hrs; ref. 10 and $3.8 \cdot 10^{-5}$ /sec, $\tau = 5.1$ hr; ref 11). In view of recent evidence that the decay rate of α -toxin-eel electroplax nAChR complexes varies as a function of incubation time and concentration of native toxin in the quench solution [22,23], these small discrepancies may reflect variation in experimental conditions or intrinsic differences in binding site configuration in membrane-bound and solubilized form. While the subcellular distribution of cortical and sub-cortical α -Bgt binding sites detected in this and other [6,23,24] investigations is inconsistent with that expected for sites located quantitatively in synaptic junctional complexes, the recent identification of α -Bgt binding sites on post-junctional membranes [24,25] sedimenting with characteristics of microsomal fraction components would appear to resolve this matter.

Consequently, it is unnecessary to resort to postulates of binding entity precursors or axonal and dendritic sites to explain the data. It is expected that judicious variation of homogenization and preparation conditions might effect significant enrichment of specific α -Bgt binding sites in particular membrane fractions.

Taken together, the high-affinity, ionic strength-insensitive, essentially irreversible nature of the binding of α -Bgt to rat brain membrane fractions, the non-uniform distribution of those binding sites, and their apparent enrichment in synaptosomal or postsynaptic-membrane fragments provide strong support for the existence of a specific α -Bgt binding component with properties resembling those expected of a post-synaptic nAChR. However, despite identification of α -Bgt binding sites in mammalian brain in vitro by histological techniques consistent with the distribution of known cholinergic markers [26,27] and in CNS synaptic structures [28], the response of known CNS cholinergic neurons to acetylcholine was found not to be blocked by α -Bgt [29,30], suggesting that α -Bgt binding sites in the CNS might be no more than a curiosity with respect to physiological function. To provide support for the identification of these sites as functional nAChR, their cholinergic nature must be demonstrated. Cholinergic ligands, given their ability to block α -Bgt binding to peripheral nAChR, might be expected to prevent, at high enough concentrations, biologically specific binding α -Bgt to non-nAChR sites. Thus, the demonstration here and elsewhere [6,7,9,10,12,24] of such competition ability is not itself sufficient evidence that CNS nAChR are the targets of α -Bgt. Based on the assumption that the kinetics of cholinergic ligand binding to either nAChR or non-nAChR is fast, and barring any effect of ligand on specific site binding properties, there should be no difference in ligand competition effectiveness

toward blocking toxin binding whether ligand is coincubated with toxin, or preincubated with nAChR prior to exposure to toxin. In contrast, if inactivation of the ligand by degradative enzymes is prevented, any alteration in binding properties induced by pretreatment with cholinergic drug would suggest that the binding entity is a cholinergic receptor, capable of undergoing dynamic change in response to specific ligand. The latter possibility is shown to be the case for the α -Bgt binding entity under study here. The magnitude of the preconditioning response, most evident for the natural ligand, ACh, and its manifestation only in the presence of cholinergic agonist, provides additional evidence that the binding entity under study is a CNS nAChR. The direction of the effect, namely, that pretreatment with agonist induces a high-affinity form toward agonist relative to toxin or antagonist, precludes the possibility that non-specific degradative or inactivation artifacts contribute to the phenomena. This evidence for the existence of a biochemical equivalent of physiological desensitization in the CNS implies that, like the counterpart in the electric organ [31], the CNS nAChR must exist in at least three states; at rest (binding sites unoccupied, channel closed), in the transitory open-channel configuration (sites occupied, channel open) and in the desensitized state (sites occupied, channel closed). The results also indicate that the receptor molecule itself is at least partially responsible for manifestation of desensitization. The CNS receptor may be distinguished from electroplax nAChR in that the desensitization phenomena is strictly in response to agonist, with a high-affinity form toward agonist resulting. In contrast, Torpedo nAChR shows conditioning effects in response to both agonist and antagonist [31]. Furthermore, the observation that all specific α -Bgt binding sites in the CNS may be displaced by both agonist and antagonist

is in contradistinction to the case for electrophysiological receptor, where agonist competes for only one-half the α -toxin sites [32]. The ineffectiveness of hexamethonium toward blocking α -Bgt binding to brain nAChR suggests that CNS and ganglionic cholinergic receptors are to be distinguished as well.

While further detailed characterization of CNS nAChR toxin-ligand interactions is necessary, the preliminary results presented here suggest that the apparent inability of α -Bgt to block central cholinergic responses might be explained by invoking the existence of ineffective toxin binding at some agonist sites. That other α -toxins can block CNS cholinergic responses [30] suggests that there are active site structural differences in these agents relative to those for α -Bgt.

In the interest of purifying this CNS nAChR, the preliminary solubilization data would indicate that this entity is partially resistant to detergent dissolution. The extent of purification and recovery of Emulphogene solubilized α -Bgt binding sites are in close agreement with comparable experiments by Moore and Brady [33], but our results do not compare with reports on Triton X-100 solubilization experiments [10,11]. Nevertheless, the unusual α -toxin binding properties exhibited by detergent solubilized α -Bgt binding sites may indicate that irreversible alteration of receptor properties occurs on its removal from the native membrane environment.

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Table I
 α -Bgt Binding

Radiolabeled α -Bgt derivative	B _{max} (fmole/mg)	app K _D (nM α -Bgt)
[¹²⁵ I] ₁] α -Bgt	41.3 $\pm$ 3.4	1.81 $\pm$ 0.31
[³ H] α -Bgt	36.9 $\pm$ 4.6	1.65 $\pm$ 0.42

Average ($\pm$ standard error) of nine determinations of maximum specific binding (B_{max}, fmole/mg) and apparent K_D (nM α -Bgt) for binding of [³H] α -Bgt and [¹²⁵I]₁] α -Bgt to crude mitochondrial fraction membranes. Values determined from linear regression analysis of double reciprocal plots under conditions as described in Fig. 2.

TABLE II

Subcellular Distribution

<u>fraction</u>	<u>relative activity (mol/mg)</u>
<u>nuclear</u>	
0-1000 g pellet	1.0
<u>"mitochondrial"</u>	
1000- 9000 g pellet	1.5
1000-13 000 g pellet	1.8
1000-17 800 g pellet	1.9
<u>"microsomal"</u>	
9000 g supernatant	2.3
13 000 g supernatant	2.3
17 800 g supernatant	0.9

Comparison of specific α -Bgt binding to membranes derived from crude subcellular fractions differing in sedimentation characteristics in 0.32M sucrose. All fractions resuspended in binding Ringers, assays carried out at final salt concentration as indicated in Fig. 2. A cold chase and 4 cycle wash (2.5 hrs) routine was performed for particulate fractions assays, and Millipore HAWP filtration technique was used for microsomal fractions.

TABLE III

Summary of Agonist, Antagonist Competition Effectiveness

Competitor	IC ₅₀		Ratio co/pre
	Coincubation	Preincubation	
Acetylcholine	$\sim 10^{-4}$	2.5×10^{-6}	~ 40
Carbamylcholine	9×10^{-5}	1.4×10^{-5}	6.4
Nicotine	5×10^{-6}	1.5×10^{-6}	3.4
α -Bungarotoxin	6×10^{-10}	4×10^{-10}	1.5
d-tubocurarine	4×10^{-5}	4×10^{-5}	1.0
atropine	$> 10^{-3}$	$> 10^{-3}$	-
hexamethonium	$> 10^{-3}$	$\sim 10^{-3}$	-

Data taken from Fig. 6 for effectiveness of drugs toward blocking [$^{125}\text{I}_1$] α -Bgt sites in CMF. IC₅₀ values determined directly from Fig. 6.

TABLE IV

Summary of Solubilization Experiments

Detergent	soluble		SA	insoluble		
	% prot	% α -Bgt sites		% prot	% α -Bgt sites	SA
None	0	0	-	100	100	1.00
Triton X-100	66	56	0.85	34	44	1.29
Triton X-100 +1% azide + 0.1mM PMSF	62	61	0.98	38	39	1.03
Tween 20	16	15	0.94	84	85	1.01
Tween 80	12	12	1.00	88	88	1.00
Emulphogene	41	54	1.32	59	46	.82
Deoxycholate	40	78	1.95	60	22	.37

Effectiveness of detergents toward solubilizing membrane bound α -Bgt sites from rat brain. Crude mitochondrial fraction membranes are carried through assays for total and non-specific binding at 10nM [125 I] $_1$ α -Bgt according to protocol. Membranes are subsequently treated with 1% detergent in 5mM NaPO₄, pH 7.6, 0.1mM PMSF, 0.1% azide for 1 hour at 20°. Insoluble material is sedimented at 39 000 g for 30 min and radioactivity of pellets is determined. Filtration for specific binding in supernatant yields similar results whether filtered as ammonium sulfate insoluble matter on Millipore HAWP or directly on Whatman DE8I discs. In no case was the recovery of specific

α -Bgt sites in combined soluble and insoluble fractions less than 90% of control values. Soluble and insoluble proteins were determined by modified Lowry procedures (see Methods). Recovery of protein and α -Bgt sites, and relative specific activity (SA, mol/mg protein) are given for solubilization conditions as indicated. Average results of three different experiments are summarized.

LEGENDS TO FIGURES

- Fig. 1. Binding saturation curve for [^3H] α -Bgt binding to rat brain crude mitochondrial fraction membranes. Total ($\otimes$) and non-specific (X) binding (fmol/mg) are plotted against [^3H] α -Bgt concentration (nM). Assay volume of 750 μl contained 78mM NaCl, 3.4mM KCl, 1.3mM CaCl_2 , 0.91mM MgSO_4 , 22mM Tris-base, pH 7.4 with HCl, 0.54 mg/ml bovine serum albumin, 2.6 mg/ml CMF protein and [^3H] α -Bgt as indicated. Native α -Bgt is added to total binding samples after 60 min incubation with [^3H] α -Bgt, and an equal volume of water is added to non-specific samples, bringing final native α -Bgt concentration to 4.4 μM for all samples, non-specific samples having been preincubated (30 min) with native toxin before addition of [^3H] α -Bgt. Cold chase duration is 30 min, and centrifugation-wash routine was for 150 min.
- Fig. 2. Specific binding (total minus non-specific) of [^3H] α -Bgt to CMF (fmol/mg) plotted against free α -toxin concentration (nM, open symbols). Data are from Fig. 1 ($\otimes$, X) and from a similar experiment with different preparations of [^3H] α -Bgt and CMF (o, $\bullet$) in final assay volume of 750 μl containing 77mM NaCl, 3.3mM KCl, 1.2mM CaCl_2 , 0.91mM MgSO_4 , 22mM Tris-base, pH 7.4 with HCl, 4.4 μM native α -Bgt, 0.47 mg/ml albumin 2.6 mg/ml CMF protein and [^3H] α -Bgt as indicated. Insert shows double reciprocal plot (closed symbols) of 1/ α -toxin bound (mg/fmol) against 1/free toxin concentration (nM $^{-1}$). From linear regression analysis, the ordinate and abscissa intercepts yield values for the maximum binding capacity of 38.5 ± 6.4 fmol/mg and apparent K_D of 1.8 ± 0.2 nM α -Bgt, ($\pm$ standard error) respectively. Cold chase and wash routine as in Fig. 1.

Fig. 3. Hill plot of α -Bgt binding to CMF. Bound α -Bgt sites/unoccupied sites are plotted on a logarithmic scale against free toxin concentration, also on a logarithmic scale, for data shown in Fig. 2. Least squares linear regression analysis yields values of n ($\pm$ standard deviation) equal to 1.39 ± 0.08 for all the data (dashed line), 1.16 ± 0.09 for α -Bgt = $[0.6 - 12\text{nM}]$, and 1.93 ± 0.42 for α -Bgt = $[0 - 0.6\text{nM}]$.

Fig. 4. Kinetics of formation and decay of α -Bgt-CMF complexes. Total (o) and non-specific (●) binding of $[^{125}\text{I}]$ α -Bgt (cpm) are plotted as a function of duration of exposure to 10nM radiolabeled toxin (min). Assays in 46mM NaCl, 2mM KCl, 0.72mM CaCl_2 , 0.53mM MgSO_4 , 13.2mM Tris base, pH 7.4 with HCl, 1 mg/ml albumin, 1 mg/ml CMF protein, $10\text{ }\mu\text{M}$ native α -Bgt. Calculated rate constant for formation of toxin-CMF complex is $4.97 \pm 0.32 \cdot 10^6/\text{min}\cdot\text{mole}$ ($\tau = 14.1\text{ min}$) as determined by linear regression analysis of data plotted as $\log\%$ free against time. Displacement of specific binding ($\log\%$ α -Bgt sites bound) at 10nM $[^{125}\text{I}]$ α -Bgt against time chased with $10\text{ }\mu\text{M}$ native α -Bgt (hrs) is plotted in insert for two similar experiments (83,0). Pooled data yield calculated decay rate constant of $1.87 \pm 0.19 \cdot 10^{-4}/\text{min}$ ($\tau = 62.3\text{ hrs}$). Assay conditions as above and in Fig. 2.

Fig. 5. Buffer and ionic strength effects on α -Bgt binding to whole brain membranes. Total (open symbols) and non-specific (closed symbols) $[^3\text{H}]$ α -Bgt sites bound (dpm) at 10nM α -toxin are plotted against salt concentration on a logarithmic scale (M). For Tris experiment (lower panel) mitochondrial fractions were washed and resuspended in 5mM Na_2HPO_4 , pH 7.4, 0.1mM PMSF, 0.1% NaN_3 . Concentrations of

Tris were varied as indicated in the presence of 1.67mM Na_2HPO_4 , pH 7.4, 33 μM PMSF, 0.03% NaN_3 , 1.8 mg membrane protein 25 μM native α -Bgt. Mitochondrial fractions were washed and resuspended in 2mM Tris, pH 7.4 for subsequent experiments with varying concentrations of MgSO_4 and CaCl_2 (upper panel) and NaCl and KCl (lower panel), using 1.6 mg CMF protein. Thirty minute cold chase and wash regimen (two cycles, 1.25 hr) preceded determination of radioactivity in residual pellet.

Fig. 6. Competition of cholinergic drugs toward $[^{125}\text{I}]_1\alpha$ -Bgt binding to CMF. Specific toxin sites bound (percent) are plotted against concentration of inhibitor (M) on a logarithmic scale. Upper panel-coincubation: 2 mg CMF is added to solution containing drug at concentrations indicated and 10nM $[^{125}\text{I}]_1\alpha$ -Bgt. Lower panel-preincubation: CMF (2 mg) is preincubated with drug at 110% concentration indicated for 30 min before α -Bgt added to start reaction. (✱) native α -Bgt, ($\diamond$)-d-tubocurarine, (O)-atropine, ($\square$)-hexamethonium, ($\bullet$)-acetylcholine ($\blacktriangle$)-carbamyl choline, ($\blacksquare$)-nicotine. Assay conditions and chase and wash routine as in Fig. 4.

Fig. 7. Saturation curve for binding of $[^{125}\text{I}]_1\alpha$ -Bgt to Triton X-100 solubilized CMF. Specific binding (fmol/mg) is plotted against free toxin concentration (nM). Crude mitochondrial fraction membranes are treated with 1% Triton X-100, 10mM NaH_2PO_4 , pH 7.6 for 1 hr at 0°C, and insoluble material is sedimented at 20 000g for 30 min prior to binding assay and DE81 filtration as described in Methods. Results are from two preparations, as indicated by different symbols.

RAT BRAIN CRUDE MITOCHONDRIAL FRACTION MEMBRANES

XBL 7710-4637

Fig. 1
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RAT BRAIN CRUDE MITOCHONDRIAL FRACTION MEMBRANES

Fig. 2
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HILL PLOT OF α -Bgt BINDING TO CMF MEMBRANES

XBL 7710-4639

Fig. 3
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RAT BRAIN CRUDE MITOCHONDRIAL FRACTION MEMBRANES

XBL 7710-4634

Fig. 4
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IONIC STRENGTH EFFECTS RAT BRAIN CRUDE MITOCHONDRIAL FRACTION MEMBRANES

XBL 7710 - 4636

Fig. 5
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Fig. 6
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XBL 7710-4649

Fig. 7
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