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Interaction of Nicotinic Receptor Affinity Reagents with Central Nervous System α-Bungarotoxin-Binding Entities

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SUMMARY

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Membrane-bound α-bungarotoxin-binding entities derived from rat brain are found to interact specifically with the affinity reagents maleimidobenzyltrimethylammonium (MBTA) and bromoacetylcholine (BAC), originally designed to label nicotinic acetylcholine receptors from electroplax and skeletal muscle. Following treatment of membranes with dithiothreitol, all specific toxin binding sites are irreversibly blocked by reaction with MBTA or BAC. Affinity reagent labeling of dithiothreitol-reduced membranes is prevented (toxin binding sites are not blocked) by prior alkylation with *N*-ethylmaleimide, by prior oxidation with dithiobis(2-nitrobenzoic acid), or by incubation with neurotoxin. Reversibly associating cholinergic agonists and antagonists retard the rate of affinity reagent interaction with toxin receptors. The apparent rates of affinity reagent alkylation of toxin receptors, and the influences of other sulfhydryl/disulfide reagents on affinity labeling are comparable to those observed for reaction with nicotinic acetylcholine receptors in the periphery. The results provide further evidence that central nervous system α-bungarotoxin receptors share a remarkable number of biochemical properties with nicotinic receptors from the periphery.

INTRODUCTION

As a first step in understanding mechanisms of higher order-central nervous system (CNS)[2] function, characterization of the properties of CNS neurotransmitter receptors offers considerable promise. The availability of peripheral tissues highly enriched in nAChR, as derived from electric organs of ray and eel, and the discovery that curaremimetic neurotoxins from poisonous snakes bind to nAChR with high affinity and specificity (1), have allowed much progress to be made toward elucidation of biochemical properties of peripheral nAChR (2).

α-Bungarotoxin, derived from the venom of *Bungarus multicinctus*, has been shown to interact specifically, and with high affinity, with membrane-bound sites derived from rat brain (3). Nevertheless, the physiological relevance of CNS α-BgtR has been questioned on the basis of apparent impotency of α-Bgt as an antagonist at certain central (e.g., (4), but see (5, 6)) and autonomic (e.g., (7, 8), but see (9)) cholinergic synapses, despite a wide body of pharmacological, histological and biochemical evidence consistent with their identity as true CNS nAChR (see 10). Toward resolution of this paradox, recent studies have shown that the affinity of CNS α-BgtR for cholinergic agonist is sensitive to exposure to agonist, modification of receptor sulfhydryl/disulfide groups, and the presence of Ca^{2+} (11, 12). Similar responses of peripheral nAChR to such manipulations have been described (13–20), suggesting striking biochemical similarity between CNS α-BgtR and peripheral nAChR.

In order to provide further biochemical evidence in favor of identity of CNS α-BgtR as authentic nAChR, we have undertaken a study of the interaction of CNS α-BgtR with affinity reagents designed to specifically label nAChR in skeletal muscle and electroplax (21).

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[2] The abbreviations used are: CNS, central nervous system; nAChR, nicotinic acetylcholine receptor; α-Bgt, α-bungarotoxin; [^{3}H]-α-Bgt, ^{3}H-labeled α-Bgt; α-BgtR, α-Bgt receptor; MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide; BAC, bromoacetylcholine bromide; α-BgtR-SH, reduced α-BgtR active site sulfhydryl; nAChR-SH, reduced nAChR active site sulfhydryl; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; K_{30}, concentration of affinity reagent necessary to reduce the number of specific [^{3}H]-α-Bgt–receptor complexes formed after 30 min incubation to 50% of control values; ACh, acetylcholine; d-TC, *d*-tubocurarine; β-ME, β-mercaptoethanol.

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These reagents combine the features of two molecules. One part consists of a moiety resembling a cholinergic ligand, including a quaternary ammonium ion, which reacts at the negative subsite of the active site of the receptor, and renders specificity to the reagent. The other portion of the molecule contains a reactive center that alkylates sulfhydryl groups. If the geometry of the reagent is such that the alkylating center comes into juxtaposition with a reduced sulfhydryl on the receptor when the quaternary ammonium group is bound at the negative subsite of nAChR, the reagent will alkylate reduced nAChR with much higher specificity, and at a faster rate than it will react nonspecifically with "non-receptor" SH. Two such reagents are maleimidobenzyltrimethylammonium and bromoacetylcholine, developed by Karlin and associates (see 21–24). These reagents alkylate reduced nAChR with high affinity, irreversibly antagonize (MBTA) or activate (BAC) the flux of ions through the receptor-coupled channel at peripheral cholinoceptive synapses of eel electroplax, and perturb curaremimetic neurotoxin binding to nAChR. Our reasoning is that if CNS α-BgtR reacts with these affinity reagents, the active site of the receptor must bear remarkable resemblance, at the molecular level, to authentic nAChR in the periphery, thereby providing strong evidence for identity of CNS α-BgtR as nAChR.

EXPERIMENTAL PROCEDURE

Methods for purification of α-Bgt from crude lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium), and for preparation of [^{3}H]-α-Bgt (specific activity of 25 dpm/fmol, 95% bound by excess nAChR from *Torpedo californica* electroplax), were as previously described (25–28).

Membrane fractions. Crude mitochondrial fraction membranes were prepared fresh daily from brain (cerebellum was discarded) of Wag/Rig rats (Lawrence Berkeley Laboratory rat colony, killed by decapitation). Unless otherwise noted, all manipulations were at 0–4°. A 10% (v/v) homogenate was prepared in 0.32 M sucrose, 0.5 mM NaH_2PO_4, pH 7.5, 10 μM phenylmethylsulfonyl fluoride, 0.1% NaN_3 with 10 strokes of a Teflon pestle rotating at 1000 rpm (Sunbeam) within a Pyrex homogenizer. Crude nuclear fractions and cellular debris were removed by centrifugation at 2000*g* for 15 min. Supernatants were then pooled and subjected to centrifugation at 28,000*g* for 15 min (Sorvall RC-2B, SM-24 rotor). The pellet was resuspended in binding Ringer's medium (115 M NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 μM phenylmethylsulfonyl fluoride, 0.1% NaN_3, 33 mM Tris, pH 7.6 with HCl), sedimented at 38,000*g* for 15 min and resuspended. Samples were then either divided into aliquots for binding assays, or were subjected to treatment with thio-group reagents prior to binding assays as described below or in figure and table legends. These preparations typically contained 15–25 fmol toxin sites/mg membrane protein, and 12–18 mg protein/ml.

Thio-group modification. In general, membrane preparations at ~15 mg/ml were treated at 0–4° with 0.3 mM DTT (Calbiochem), DTNB (Aldrich) or $Na_2S_2O_5$ (Baker) for 20 min, or with 0.3 mM NEM (Sigma) for 5 min. In all cases, thio-group reagent-treated membranes were diluted in the appropriate Ringer's medium and subjected to centrifugation at 38,000*g* for 5–10 min to remove excess reagent prior to subsequent chemical treatment or preparation of samples for binding assays. Modifications of this general procedure and the precise sequence of chemical treatments are described in figure or table legends.

Affinity reagents. MBTA, synthesized according to Karlin (22) and stored in acetonitrile, was a gift from Dr. Mark G. McNamee. BAC was synthesized as described by Damle *et al.* (23) and stored in crystalline form. Immediately prior to use, stock solutions of MBTA were dried *in vacuo* and dissolved in ice-cold 0.1 mM HCl. The concentration of MBTA was determined by optical absorbance (22). Crystalline BAC was weighed on a microbalance and dissolved in ice-cold 0.1 mM HCl to the desired final concentration.

Affinity labeling was generally carried out by addition of 10 μl MBTA or BAC at the appropriate concentration to 200 μl of resuspended membranes, treated as described above or in figure and table legends. Reactions with BAC were carried out in the presence of 100 μM eserine (Calbiochem) in order to block cholinesterase activity. Unless otherwise noted, the reaction was quenched after 5 min by dilution in 5 ml of Ringer's medium containing 20 μM DTT. A variety of other quench procedures were tested and not found to affect the data. Following centrifugation for 10 min at 38,000*g*, pellets were resuspended with 160 μl of buffer, and subjected to α-toxin binding assays. For experiments examining the effect of affinity reagent on toxin binding rate, affinity labeling was carried out batchwise by addition of 100 μl reagent to 2.5-ml aliquots of membrane suspension. The reaction was quenched by addition of 12.5 ml Ringer's medium containing 20 μM DTT, 38,000*g* pellets were resuspended to 2.5 ml, and 200-μl aliquots were distributed for toxin binding assays.

α-Toxin binding assays. The extent of specific [^{3}H]-α-Bgt binding to membranes represents the difference in radioactivity bound to test samples exposed to 0.4 μM native toxin for 25 min following treatment with 10 nM [^{3}H]-α-Bgt (250 μl final volume) relative to blank samples pretreated with native toxin for 30 min prior to exposure to [^{3}H]-α-Bgt. The native toxin chase period serves to terminate [^{3}H]-α-Bgt specific binding as well as to permit nonspecific and short-lived pseudospecific binding of [^{3}H]-α-Bgt to equilibrate to values for native toxin-pretreated blank samples. To determine reversible effects of BAC or MBTA on toxin-receptor interaction, levels of specifically bound [^{3}H]-α-Bgt were determined at a variety of affinity reagent concentrations. Irreversible effects were assessed by toxin binding assay after treatment of membranes as described above. The concentration of reagent necessary to reduce specific binding of [^{3}H]-α-Bgt by 50% over a 30-min incubation with toxin (K_{30} value) was assumed to equal the concentration of reagent necessary to occupy 50% of α-BgtR. For toxin binding rate studies, the incubation period was varied from 0 min to 20 hr. Following the native toxin chase period, membranes were subjected to two cycles of suspension in 3 ml of Ringer's medium and centrifugation for 15 min at 38,000*g*. Supernatants were removed, pellets were drained to dryness and then resuspended and quantitatively transferred to vials containing Aquasol-2 (New

England Nuclear) or 3a70B cocktail (Research Products International) for ^3H determination by liquid scintillation counting (Packard Tri-Carb 3375 or Beckman LS 9000; 25–30% efficiency). Typically, ~300 cpm were specifically bound in the absence of cholinergic ligand out of ~500 total cpm. All assays were carried out at 21° with shaking.

Other techniques. Membrane protein concentration was determined by the method of Lowry *et al.* (29), with the modification that membranes were dissolved for 30 min in NaOH-bicarbonate supplemented with 1% sodium dodecyl sulfate.

Concentrations of membrane-bound sulfhydryl groups were determined by reaction with DTNB in the presence of sodium arsenite (30), against a cysteine standard curve.

RESULTS AND DISCUSSION

When CNS α-BgtR is exposed to varying concentrations of DTT or β-ME, or when DTT (300 μM) treated α-BgtR is subjected to oxidation with DTNB (data not shown) or alkylation with NEM (Fig. 1), interaction of toxin with α-BgtR is perturbed only at reagent concentrations well in excess of 1 mM, where non-specific alterations in α-BgtR and/or α-Bgt binding are likely to occur. Therefore, for α-BgtR treated according to routine protocol with 300 μM or 20 μM DTT, β-ME, DTNB or NEM, no permanent effects on toxin binding are manifest.

The ability of MBTA and BAC to inhibit α-Bgt binding to membrane-bound α-BgtR, as shown in Fig. 1, varies as a function of SH modification preceding exposure to affinity reagent. When MBTA or BAC are exposed to α-BgtR reduced and alkylated/oxidized by appropriate chemical modification as described under Experimental Procedure, they exhibit poor ability to inhibit toxin binding (Fig. 1). The number of toxin–receptor complexes formed over 30 min incubation are diminished to one-half of control values in the presence of ~10 mM MBTA or ~400 μM BAC in the displayed experiment. These concentrations of affinity reagent thus serve as a crude estimate of the affinity of reversibly associating MBTA or BAC for α-BgtR, as irreversible reaction with prealkylated/preoxidized α-BgtR is prevented. The toxin binding inhibition potencies of MTBA and BAC are increased markedly when reagents interact with DTT-reduced α-BgtR. Concentrations of affinity reagent necessary to block one-half of toxin binding over a 30 min time course (K_{30}) in this representative experiment are ~1 μM for MBTA, and ~3 μM for BAC. These results demonstrate that free receptor SH must be available for high affinity reaction with affinity reagent. It should be noted that inhibition of toxin binding is complete and that the data are consistent with a homogeneous population of sites reacting with toxin and affinity reagent. Extensive washing of reduced and affinity reagent-reacted α-BgtR does not affect K_{30} values, even when washing proceeds in the presence of DTT, β-ME, DTNB or NEM. Therefore, inhibition of toxin binding appears to be irreversible, and is presumably mediated by alkylation of α-BgtR-SH with affinity reagent. The K_{30} values determined in these experiments represent upper limits for the concentration of MBTA or BAC necessary to occupy one-half of the α-BgtR-SH sites. While the rate constant for specific alkylation of peripheral nAChR-SH with affinity reagent is several orders of magnitude larger than the rate constant for nonspecific alkylation of non-nAChR-SH (22), in these crude membrane preparations the concentration of non-α-BgtR-SH is ~125 μM prior to and ~190 μM following DTT treatment (Table 1) compared to ~250 pM α-Bgt-SH (determined from quantities

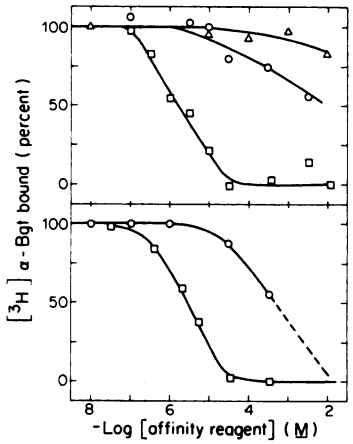

FIG. 1. *Blockade of [^3H]-α-Bgt binding by reaction of brain membranes with cholinergic receptor affinity reagents*

Levels of [^3H]-α-Bgt specifically bound to membranes are plotted against the molar concentration (logarithmic scale) of MBTA (upper panel) or BAC (lower panel) present in the reaction mixture. Membranes were treated with DTT (□, both panels) DTT-NEM (○, lower panel) or DTT-DTNB (○, upper panel), as described under Experimental Procedure, prior to addition of affinity reagent. Also illustrated is the effect of DTT-NEM treatment on specific [^3H]-α-Bgt binding (△, upper panel). Data points represent single determinations, and are typical of numerous replicate experiments.

TABLE 1

Determination of membrane-bound sulfhydryl groups

Free sulfhydryl groups in rat brain membranes were assayed by reaction with DTNB in the presence of 300 μM sodium arsenite (30) to minimize reaction with residual DTT. Aliquots (500-μl) of membrane suspension were reacted with: (a) 1.2 mM NEM, washed three times by dilution in 12 ml of Ringer's medium, centrifugation at 38,000*g*, and resuspension to 500 μl, and reacted with 350 μM DTT (NEM–DTT); (b) 350 μM DTT (DTT); or (c) buffer only (none). All samples were diluted to 12 ml, sedimented at 38,000 *g* to remove excess DTT, and resuspended in 500 μl of Ringer's medium. One-half of the DTT-treated samples and one-half of the untreated samples were further reacted with 1.2 μM NEM. All samples were subsequently diluted to 1.5 ml and brought to 300 μM sodium arsenite. 100 μl of 4.4 mM DTNB was added to each sample. Following sedimentation at 38,000 *g*, supernatants were read for optical absorbance at 412 nm, and compared to a standard curve derived from DTNB reaction with cysteine. Optical absorbance of DTT–NEM-treated membranes was subtracted from other DTT-treated samples, and native membranes treated with NEM were used as blanks for untreated membrane samples. Concentration of SH in untreated samples represents the concentration of native sulfhydryls. Concentration of SH in NEM–DTT-treated samples represents twice the concentration of native disulfides. Concentration of SH in DTT-treated samples represents the sum of [native SH] and 2x [native S-S].

Treatment	Concn of SH (μM)
NEM–DTT	65
DTT	190
None	125

of toxin sites). Thus K_{30} values represent the concentration of affinity reagent necessary to block one-half of toxin-binding (occupy one-half α-BgtR-SH with MBTA or BAC) before reagent is consumed by nonspecific interaction with non-α-BgtR-SH. It is not surprising, then, that the results are essentially the same whether the reaction with affinity reagent is allowed to go to completion (occurs within 5 min exposure) before dilution and centrifugation, or quenched with excess DTT, β-ME, DTNB, or NEM prior to, or simultaneous with, dilution and centrifugation. Quantitatively, since 0.5% of non-α-BgtR-SH is labeled at 1 μM MBTA or BAC, while 50% α-BgtR-SH is labeled, the rate constant for labeling of α-BgtR-SH is ~140-fold larger than that for nonspecific labeling of non-α-BgtR-SH in this preparation. It is unclear whether the ratio of rate constants for specific versus nonspecific labeling may be increased by improvements in the purity of α-BgtR in brain membranes.

In order to further demonstrate irreversibility of the reaction of MBTA or BAC with DTT-reduced α-BgtR, effects of affinity reagent treatment on the rate of specific [^{3}H]-α-Bgt binding to α-BgtR were assessed (Fig. 2). The results show that the extent of inhibition of specific toxin binding at a given concentration of MBTA or BAC is invariant, whether toxin and α-BgtR are incubated for 1 min or 20 hr. This further supports the interpretation that MBTA and BAC irreversibly alkylate α-BgtR and thereby block toxin binding. Moreover, the rate constant for toxin binding to sites not blocked by affinity reagent alkylation is essentially the same as the rate constant for toxin binding to α-BgtR in the absence of inhibitor.

If the site of interaction of MBTA and BAC with α-BgtR is to be related to the receptor active site, one would expect that cholinergic agonists and antagonists might perturb the rate of MBTA or BAC-alkylation of α-BgtR-SH. In pilot studies, the half-time of affinity alkylation was found to be ~30 sec for BAC (at 3 μM) and less than 10 sec for MBTA (at 3 μM), which, together with other sensitivity limitations, precluded precise study of reaction rates directly using our experimental designs. Nevertheless, another estimate of the effects of reversibly associating cholinergic ligand could be made by examining the concentration dependence of MBTA or BAC blockade of toxin binding in the presence and absence of ligand. Reasoning that only the specific interaction of MBTA or BAC with α-BgtR-SH would be perturbed as receptor sites are occupied by ligand, and that consumption of affinity reagent by reaction with non-α-BgtR-SH would proceed unaffected, such experiments might yield relative alkylation rate information when reaction with affinity reagent is restricted to short incubation periods (30 sec) and quenched by dilution in the presence of 20 μM DTT. One limitation is that it would be necessary to reduce the rate of specific alkylation at least threefold to have adequate sensitivity. Consideration also was given to the fact that the affinity reagent reaction is irreversible. It is likely that only a few affinity reagent–receptor collision complexes need be formed before irreversible attachment occurs, while many reversible ligand–receptor interactions need take place to occlude the active site. One additional complication is that the affinity of cholinergic agonist for reduced α-BgtR is very much lower than that for oxidized α-BgtR (12).

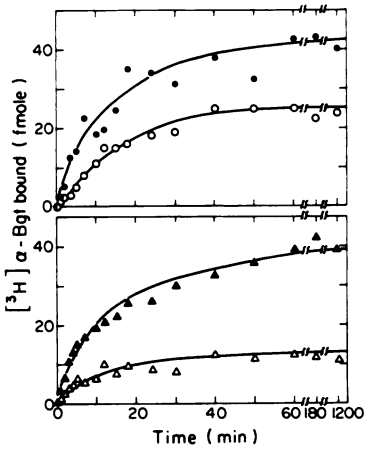

FIG. 2. *Modification of toxin binding rate by treatment of membranes with affinity reagent*

Levels of specifically bound [^{3}H]-α-Bgt (fmol) are plotted as a function of time (min) for membranes treated batchwise with DTT only (●, ▲), with DTT–1 μM MBTA (○), or with DTT–2 μM BAC (△). Details of toxin binding assay and batchwise treatment of membranes with affinity reagent are given under Experimental Procedure. Data points represent single determinations.

Despite these difficulties, results illustrated in Fig. 3 demonstrate that, at high enough concentrations, cholinergic agonists and antagonists do retard the rate of alkylation of α-BgtR-SH by MBTA/BAC. In this experiment, in the absence of ligand, K_{30} values are ~3 μM for MBTA and 3 μM for BAC. At 200 mM ACh (100 times the concentration of ACh necessary to block one-half of toxin binding to DTT-reduced α-BgtR; see Ref. (12)), K_{30} values are increased 3-fold to ~10 μM for MBTA; K_{30} value of ~50 μM is found for BAC, a 20-fold increase relative to the control value. As the affinity of d-TC for α-BgtR is largely unaffected by thio-group manipulation (12), and d-TC dissociates from α-BgtR more slowly than ACh,[3] lower concentrations of d-TC are effective in perturbing affinity reagent alkylation of α-BgtR-SH. At 4 mM, d-TC raises K_{30} values to ~50 μM for MBTA, and ~600 μM for BAC. That is, at d-TC concentrations 200 times higher than that necessary to block one-half toxin binding, there is observed 20-fold and 200-fold increases in K_{30} values for MBTA and BAC, respectively.

Provisionally, results of limited experiments are consistent with nicotinic pharmacology of the MBTA binding site. Nicotine at 5 mM, decamethonium at 3 mM, and d-TC at 2 mM are the most potent inhibitors of MBTA labeling of toxin receptors, causing a 10-fold increase in MBTA values relative to controls. Corresponding concentrations of nicotine, decamethonium and d-TC necessary to block one-half of α-Bgt binding to DTT-reduced α-BgtR are 10, 40 and 20 μM (12). At approximately 2 mM, atropine or hexamethonium block one-half of specific interaction of [^{3}H]-α-Bgt with reduced α-BgtR (12), but 100 mM atropine or hexamethonium is necessary to raise MBTA K_{30} values 10-fold. Thus, at concentrations approximately 100-fold higher than those necessary to block one-half of toxin binding to reduced α-BgtR, these

[3] Lukas, unpublished observations.

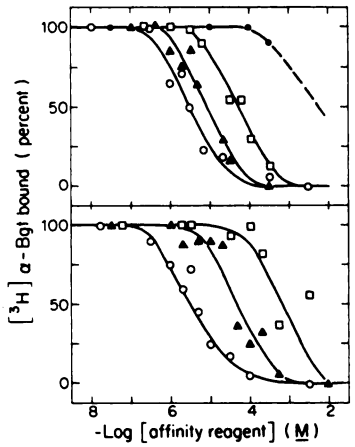

FIG. 3. *Effects of reversibly associating cholinergic ligand on efficiency of affinity labeling*

Levels of [^{3}H]-α-Bgt bound (percent) are plotted as a function of affinity reagent concentration (logarithmic scale; molar) present in the reaction mixture. Membranes were treated with DTT and subjected to reaction with MBTA (upper panel) or BAC (lower panel) in the presence of 4 mM d-tubocurarine (□) or 200 mM acetylcholine and 100 M eserine (▲). Control experiments in the absence of cholinergic ligand ○) or for DTT-reduced membranes treated with NEM prior to affinity reagent reaction (●) are also shown. Affinity reagent reaction was quenched after 30 sec by dilution as described under Experimental Procedure. Membranes were resuspended, diluted, and recentrifuged repeatedly over a 4-hr period prior to toxin binding assay in order to remove acetylcholine and *d*-tubocurarine. Data points represent single determinations, but results are typical of similar experiments.

igands retard MBTA alkylation of α-BgtR-SH about 10-old. While this relationship does break down for ACh nd carbachol, which cause only 3-fold increase in MBTA K_{30} values at 200 mM, ability of ACh and carbachol to lock MBTA labeling is increased on protracted incubation with reduced α-BgtR. This result suggests that he MBTA sites are on an entity that changes its affinity or cholinergic agonist on exposure to agonist. Neuroleptic drugs and catecholamines do not alter MBTA K_{30} alues.

Taken together, the results suggest that reversibly ssociating cholinergic ligands do retard specific reaction f MBTA or BAC with CNS α-BgtR. The fact that oncentrations of ligand necessary to block MBTA or BAC labeling of reduced α-BgtR are markedly higher han those necessary to block toxin binding does not, in tself, compromise our conclusions; such is also the case or interaction of reduced *Torpedo* electroplax nAChR vith reversibly associating ligands, toxin, and affinity eagents (Dr. McNamee, personal communication). It is ikely that potency of cholinergic ligand inhibition of ffinity alkylation reflects complex relationships between luration of receptor occupancy and steric hindrance onsiderations.

Results of experiments examining α-Bgt competition oward specific reaction of MBTA or BAC with α-BgtR ites are summarized in Table 2. Toxin-receptor complexes have a finite half-life. Hence, the number of available toxin binding sites may be measured after the original complexes have dissociated, and the effects of MBTA r BAC treatment may be assessed. As might be expected, the results indicate that toxin prevents affinity reagent labeling of reduced toxin receptors.

TABLE 2

Inhibition of affinity labeling by α-Bgt

Effects of α-Bgt on affinity labeling were determined by reacting reduced α-BgtR with α-Bgt before exposure to MBTA or BAC. Excess toxin was removed and, after a few half-lives, membranes were reacted with labeled toxin to determine the quantity of sites. Membrane fractions were treated with DTT (0.3 mM, 20 min) and divided into four sets of six samples (200-μl aliquots). One set was treated with 0.3 mM NEM for 10 min, followed by exposure to affinity reagent (DTT-NEM). One set was exposed to buffer for 10 min, followed by exposure to affinity reagent (DTT). One set was exposed to 400 μM native α-Bgt for 10 min, followed by affinity reagent treatment (DTT-α-Bgt). One set was exposed to α-Bgt only, without reaction with affinity reagent. Samples were diluted 60-fold, subjected to centrifugation at 40,000 *g* for 15 min, and resuspended in 5 ml buffer, sedimented and resuspended in 5 ml buffer. After each overnight incubation, the sedimentation/resuspension process was repeated. On Day 6 (138 hr) samples were assayed for specific [^{3}H]-α-Bgt binding (30 min incubation, four tests, two blanks each set). DTT-NEM-treated membranes bind 16.0 ± 1.3 fmol [^{3}H]-α-Bgt (0% toxin sites blocked by affinity reagent) and serve as a control for DTT samples.[a] DTT samples treated with MBTA bind 7.4 ± 1.1 fmol [^{3}H]-α-Bgt (54% blocked) and DTT samples treated with BAC bind 3.8 ± 1.8 fmol [^{3}H]-α-Bgt (76% blocked). Samples treated with native α-Bgt only bind 11.8 ± 2.3 fmol [^{3}H]-α-Bgt (consistent with 72-hr half-time for dissociation of α-Bgt-nAChR complexes; 26% toxin sites blocked by toxin, 74% toxin sites available) and serve as a control for DTT-α-Bgt samples.[b] DTT-α-Bgt samples treated with MBTA and BAC bind 11.7 ± 3.0 fmol [^{3}H]-α-Bgt (1% available toxin sites blocked by MBTA) and 11.6 ± 1.9 fmol [^{3}H]-α-Bgt (2% available toxin sites blocked by BAC), respectively.

Treatment	Percentage toxin sites blocked by 10 μM MBTA	Percentage toxin sites blocked by 10 μM BAC
DTT-NEM[a]	0	0
DTT[a]	54	76
DTT-α-Bgt[b]	1	2

Given the large excess of non-α-BgtR-SH over α-BgtR-SH available for reaction with cholinergic receptor affinity reagents, a series of experiments was conducted in an attempt to find conditions that would facilitate specific labeling of α-BgtR-SH with MBTA or BAC by reduction of nonspecific reaction with non-α-BgtR-SH. When native α-BgtR is reacted with NEM, washed free of NEM, reduced with DTT, and alkylated with affinity reagent, K_{30} values are essentially unaltered (Fig. 4). This result indicates that, although the concentration of natural SH in our preparations is more than the concentration of SH liberated after reaction with DTT, there is negligible increase in specificity of MBTA or BAC labeling by prealkylation with NEM. These results are nevertheless consistent with results of similar experiments on prealkylation and affinity labeling of *Electrophorus* electroplax nAChR *in situ* (24).

When affinity reagents (at varying concentrations) and 300 μM NEM are simultaneously added to DTT-reduced membranes, K_{30} for MBTA is essentially unaltered, but K_{30} for BAC is increased ~15-fold relative to K_{30} values in the absence of NEM (Fig. 4). In these experiments, it is not consumption of affinity reagent that determines K_{30} values, but the relative rates of labeling of α-BgtR-SH by reagent and NEM. Thus, the results reflect the larger rate constant for MBTA alkylation of α-BgtR-SH relative to that for BAC. Assuming that the rate constant for reaction of BAC with α-BgtR-SH is ~5000 M^{-1} sec^{-1}

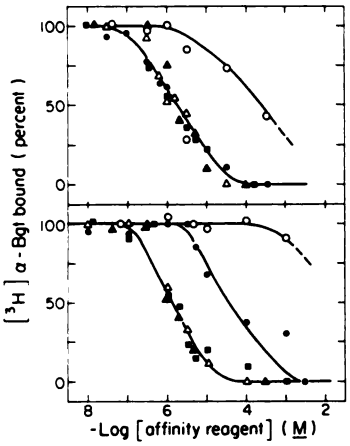

FIG. 4. *Effect of sulfydryl/disulfide group treatment on affinity labeling*

Levels of [^{3}H]-α-Bgt bound (percent) are plotted as a function of the molar concentration (logarithmic scale) of MBTA (upper panel) or BAC (lower panel). Membranes were treated with $Na_2S_2O_5$ (○), $Na_2S_2O_5$-DTT (▲), or NEM-DTT (washed free of NEM prior to DTT reaction (■), or DTT-treated membranes were added to affinity reagent alone (△) or affinity reagent plus 300 μM NEM (●). Affinity reagent reaction was quenched, and samples were subjected to toxin binding assay as described under Experimental Procedure. Data points represent single determinations, but are representative of numerous replicate experiments. For affinity alkylation in the presence of 300 μM NEM, it is assumed that the K_{30} value represents the concentration of MBTA or BAC where one-half of α-BgtR-SH are alkylated with affinity reagent, and one-half with NEM. Under these conditions, $[BAC]k_{BAC} = [NEM]k_{NEM}$, and $[MBTA]k_{MBTA} = [NEM]k_{NEM}$, where [BAC] and [MBTA] are K_{30} values (30 and 1 μM, respectively), [NEM] = 300 μM, and k_x is the rate constant for alkylation of α-BgtR-SH by species x. Assuming $k_{BAC} = 5000\ M^{-1}\ sec^{-1}$ (29), k_{NEM} and k_{MBTA} may be calculated.

and is the same for reaction of BAC with nAChR-SH from the periphery (23), the apparent rate constants for reaction with α-BgtR-SH are ~500 M^{-1} sec^{-1} for NEM and ~10^5 M^{-1} sec^{-1} for MBTA. These values are comparable to rate constants of 1620 M^{-1} sec^{-1} for NEM reaction with cysteine [and, presumably, nAChR-SH (21)]; and 2.8×10^5 M^{-1} sec^{-1} for reaction of MBTA with nAChR from the periphery (21). Thus, the results of the experiments reported here indicate reasonable agreement in rate constants for affinity alkylation of peripheral nAChR and CNS α-BgtR.

There has appeared a report of the reaction of nAChR at frog neuromuscular junction with sodium bisulfite, which presumably reacts with receptor disulfide bonds through a heterocyclic cleavage mechanism. This treatment increases the physiological response to ACh (31). As with DTNB treatment of DTT-reduced CNS α-BgtR, treatment of native CNS α-BgtR with sodium bisulfite leaves receptor in a high-affinity state toward agonist.[3] MBTA and BAC do not alkylate bisulfite-treated CNS α-Bgt with high affinity (Fig. 4), indicating that the particular sulfhydryl(s) that reacts with MBTA or BAC is either sulfonated, or blocked from alkylation with affinity reagent through steric hindrance, following bisulfite treatment. However, following DTT-reduction of bisulfite-treated membranes, full reactivity toward MBTA or BAC returns (Fig. 4). The possibility exists that two distinct S-S residues on α-BgtR are involved in oxidation–reduction state changes related to differences in affinity of α-BgtR cholinergic agonists and reaction with affinity reagents. However, physiological (31) and the present biochemical evidence may be interpreted as demonstrating equivalence of the S-S loci involved in these events.

Taken together, the results indicate that CNS α-BgtR share a number of biochemical properties with nAChR in the periphery, and provide evidence consistent with identity of CNS α-BgtR as authentic CNS nAChR.

Provided that nonspecific interaction of affinity reagents with non-α-BgtR-SH can be minimized, the evidence in this report promises that eventual identification and isolation of CNS α-BgtR will be facilitated by use of affinity reagents as originally intended, i.e., as specific labels for nAChR.

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