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Ronald J. Lukas and Edward L. Bennett

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INTERACTION OF NICOTINIC RECEPTOR AFFINITY REAGENTS WITH CENTRAL NERVOUS SYSTEM α-BUNGAROTOXIN-BINDING ENTITIES

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Nicotinic Receptor Affinity Reagents & α-Bungarotoxin Sites
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; nAChR-SH, reduced nAChR active site sulfhydryl; DTT, dithiothreitol; DTNB, 5,5'-dithio-bis-(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 minutes incubation to 50% of control values; ACh, acetylcholine; d-TC, d-tubocurarine; β-ME, β-mercaptoethanol.

Central nervous system α-BgtR are referred to as CNS nAChR throughout RESULTS AND DISCUSSION and CONCLUSIONS.
SUMMARY


Membrane-bound α-bungarotoxin-binding entities derived from rat brain are found to interact specifically with affinity reagents maleimido-benzyltrimethylnitromethane (MBTA) and bromoacetylcholine (BAC), originally designed to label nicotinic acetylcholine receptors from the peripheral nervous system. 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 alkylation with N-ethylmaleimide, by oxidation with dithio-bis-(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 peripheral nervous system nicotinic acetylcholine receptors. The results provide further biochemical evidence that central nervous system α-bungarotoxin receptors may be identified as authentic nicotinic acetylcholine receptors, which share a remarkable number of properties with nicotinic receptors from the periphery.
INTRODUCTION

As a first step in understanding mechanisms of higher order central nervous system 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 eels, 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 (4,5 but see 6,7) and autonomic (8-11, but see 12) cholinergic synapses, despite a wide body of pharmacological, histological and biochemical evidence consistent with their identity as true CNS nAChR (see 13). 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 Ca²⁺ (14,15). Similar responses of peripheral nervous system nAChR to such manipulations have been described (16-25), 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 the peripheral nervous system (26). 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 non-specifically with "non-receptor" SH. Two such reagents are maleimido-benzyltrimethylammonium and bromoacetylcholine, developed by Karlin and associates (26-36). 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 cholinceptive synapses of eel electroplax, and perturb curaremimetic neurotoxin binding to nAChR. Our reasoning is 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 $[^3H]$-α-Bgt (specific activity of 25 dpm/fmol, 95% bound by excess nAChR from Torpedo californica electroplax), are as previously described (37-40).

Membrane fractions

Crude mitochondrial fraction membranes are prepared fresh daily from brain (cerebellum is discarded) of Wag/Rig rats (Lawrence Berkeley Laboratory rat colony). Unless otherwise noted, all manipulations are at 0-4°C. A 10% v/v homogenate is prepared in 0.32 M sucrose, 0.5 mM NaH$_2$PO$_4$, 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 are removed by centrifugation at 2000 g for 15 minutes. Supernatants are then pooled and subjected to centrifugation at 28000 g for 15 minutes (Sorvall RC-2B, SM-24 rotor). The pellet is resuspended in binding Ringer's medium (115 mM 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 38000 g for 15 minutes and resuspended. Samples are then either divided into aliquots for binding assays, or are subjected to treatment with thio-group reagents prior to the binding assays as described below or in figure and table legends. These preparations typically contain 15-25 fmol toxin sites per mg membrane protein, and 12-18 mg protein/ml.
Thio-group modification

In general, membrane preparations at ~15 mg/ml are treated at 0-4°C with 0.3 mM DTT (Calbiochem), DTNB (Aldrich) or Na$_2$S$_2$O$_5$ (Baker) for 20 minutes, or with 0.3 mM NEM (Sigma) for 5 minutes. In all cases, thio-group reagent-treated membranes are diluted in the appropriate Ringer's medium and subjected to centrifugation at 38000 g for 5-10 minutes 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 (27) and stored in acetonitrile, was a gift from Dr. Mark G. McNamee. BAC was synthesized as described by Damle et al. (29), 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 (27). 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 minutes 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 minutes at 38000 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, 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 [3H]-α-Bgt binding to membranes is generally determined as the difference in radioactivity bound to samples chased with 0.4 μM native toxin for 25 minutes following treatment with 10 mM [3H]-α-Bgt (250 μL final volume) relative to samples pretreated with native toxin for 30 minutes prior to exposure to [3H]-α-Bgt. The native toxin chase period serves to terminate [3H]-α-Bgt specific binding as well as to permit non-specific and short-lived pseudo-specific binding of [3H]-α-Bgt to equilibrate to values for native toxin-pretreated blank samples. To determine effects of BAC or MBTA on toxin-receptor interaction, levels of specifically bound [3H]-α-Bgt are determined at a variety of affinity reagent concentrations. The concentration of reagent necessary to reduce specific binding of [3H]-α-Bgt by 50% over a 30 minute incubation with toxin (K₃₀ value) is assumed to equal the concentration of reagent necessary to occupy 50% of α-BgtR. For toxin binding rate studies, the incubation period is
varied from 0 minutes to 20 hours. Following the native toxin chase period, membranes are subjected to two cycles of suspension in 3 mL of Ringer medium and centrifugation for 15 minutes at 38000 g. Supernatants are removed, pellets are drained to dryness, and then resuspended and quantitatively transferred to vials containing Aquasol-2 (New England Nuclear) or 3a70B cocktail (Research Products International) for \( ^3 \text{H} \) determination by liquid scintillation counting (Packard TriCarb 3375 or Beckman LS 9000; 25-30% efficiency). Typically, \( \sim 300 \) counts per minute are specifically bound in the absence of cholinergic ligand out of \( \sim 500 \) total cpm. All assays are carried out at 21°C with shaking.

**Other techniques**

Membrane protein concentration was determined by the method of Lowry et al. (41), with the exception that membranes are dissolved for 30 minutes in NaOH-bicarbonate supplemented with 1% sodium dodecyl sulfate.

Concentrations of membrane-bound sulfhydryl groups are determined by reaction with DTNB (42) in the presence of sodium arsenite (43), against a cysteine standard curve.
RESULTS AND DISCUSSION

When CNS nAChR is exposed to varying concentrations of DTT or β-ME, or when DTT (300 μM) treated nAChR is subjected to oxidation with DTNB (data not shown) or alkylation with NEM (Fig. 1.), interaction of toxin with nAChR is perturbed only at reagent concentrations well in excess of 1 mM, where non-specific alterations in nAChR and/or α-Bgt are likely to occur. Therefore, for nAChR 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 nAChR, as shown in Fig. 1, varies as a function of SH modification preceding exposure to affinity reagent. When MBTA or BAC are exposed to nAChR reduced and alkylated/oxidized by appropriate chemical modification as described in METHODS, they exhibit poor ability to inhibit toxin binding (Fig. 1.) The number of nAChR-α-Bgt complexes formed over 30 minutes 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 nAChR, as irreversible reaction with pre-alkylated/pre-oxidized nAChR is prevented. The toxin binding-inhibition potencies of MBTA and BAC are increased markedly when reagents interact with DTT-reduced nAChR. Concentrations of affinity reagent necessary to block one-half of toxin binding over a 30 minute time course (K_{30}) in this representative
experiment are ~ 1 μM for MBTA, and ~ 3 μM for BAC. It should be noted
that inhibition of toxin binding is complete, and that the affinity
reagent concentration dependence is mono-phasic, indicating a single
'affinity' for receptor. These results demonstrate that free receptor
SH need be available for high affinity reaction with affinity reagent.
Extensive washing of reduced and affinity reagent-reacted nAChR does
not affect K₃₀ 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 nAChR-SH
with affinity reagent. The K₃₀ values determined in these experiments
represent upper limits for the 'true' affinity of MBTA or BAC for
nAChR-SH. While the rate constant for specific alkylation of peripheral
mAChR-SH with affinity reagent is several orders of magnitude larger
than the rate constant for non-specific alkylation of non-nAChR-SH (27,
28), in these crude membrane preparations the concentration of non-
nAChR-SH is ~ 125 μM prior to and ~ 190 μM following dTT treatment
(Table I), compared to ~ 250 pM nAChR-SH (determined from quantities of
toxin sites). Thus K₃₀ values represent the concentration of affinity
reagent necessary to block one-half of toxin binding (occupy one-half
nAChR-SH with MBTA or BAC) before reagent is consumed by non-specific
interaction with non-nAChR-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 minutes expo-
sure) before dilution and centrifugation, or quenched with excess dTT,
β-ME, DTNB, or NEM prior to, or simultaneous with, dilution and centri-
fugation. Quantitatively, since 0.5% of non-nAChR-SH are labeled at 1 μM MBTA or BAC, while 50% of nAChR-SH are labeled, the rate constant for labeling of nAChR-SH is ~140-fold larger than that for non-specific labeling of non-nAChR-SH in this preparation. It is unclear whether the ratio of rate constants for specific vs. non-specific labeling may be increased by improvements in the purity of nAChR in brain membranes.

One other feature of the data in Fig. 1 emerges on examination of experimental results when affinity reagent is reacted with 'native' membranes, not exposed to other sulfhydryl/disulfide reagents. The results indicate that there is a biphasic concentration dependence of affinity reagent inhibition of toxin binding. Approximately one-third of the receptor sites in this particular preparation exist in a reduced state. That is, ~30% of toxin binding is inhibited with a high affinity \( K_{30} \) of ~1 μM BAC or MBTA. One-half of the remaining ~70% of toxin binding is inhibited at concentrations of affinity reagent of ~4 mM (MBTA) and 300 μM (BAC), in agreement with \( K_{30} \) values for low affinity reaction of MBTA or BAC with alkylated/oxidized nAChR. It should be indicated that there is considerable variation in the proportion of 'native' receptor in a reduced state ranging from 0-35% as assessed by affinity reagent reaction (but usually 0-5%). The experiment illustrated in Fig. 1 is from a preparation with unusually high levels of pré-existing reduced nAChR.

In order to further demonstrate irreversibility of the reaction of MBTA or BAC with DTT-reduced nAChR, effects of affinity reagent treatment on the rate of specific \(^3\)H-α-Bgt binding to nAChR were assessed
(Fig. 2). The results show that the concentration of MBTA or BAC necessary to block one-half of specific toxin binding is invariant, whether toxin and nAChR are incubated for 1 minute, or 20 hours. This further supports the interpretation that MBTA and BAC irreversibly alkylate nAChR and thereby block toxin binding. However, 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 nAChR in the absence of inhibitor.

If the site of interaction of MBTA and BAC with nAChR is to be related to the receptor active site, one might expect that cholinergic agonists and antagonists might perturb the rate of MBTA or BAC-alkylation of nAChR-SH. In pilot studies, the half-time of affinity alkylation was found to be ~30 seconds for BAC (at 3 μM) and less than 10 seconds 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 nAChR-SH would be perturbed, and that consumption of affinity reagent by reaction with non-nAChR-SH would proceed unaffected, such experiments might yield relative alkylation rate information when reaction with affinity reagent is restricted to short incubation periods (30 seconds) and quenched by dilution in the presence of 20 μM DTT. One limitation is that is would be necessary to
reduce the rate of specific alkylation at least 3-fold to have adequate sensitivity. Consideration also was given to the fact that affinity reagent reaction is irreversible, and it is likely that only a few 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 nAChR is very much lower than that for oxidized nAChR (15).

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 nAChR-SH by MBTA/BAC. In this experiment, in the absence of ligand, $K_{30}$ values are $\sim 3 \mu M$ for MBTA and $\sim 3 \mu M$ for BAC. At 200 mM ACh (100 times the concentration of ACh necessary to block one-half of toxin binding to DTT-reduced NAChR), $K_{30}$ values are increased 3-fold to $\sim 10 \mu M$ for MBTA and $K_{30}$ of $\sim 50 \mu M$ is found for BAC, a 20-fold increase relative to control values. As the affinity of d-TC for nAChR is largely unaffected by thio-group manipulation (15), and d-TC dissociates from nAChR more slowly than ACh (Lukas, unpublished observations), lower concentrations of d-TC are effective in perturbing affinity reagent-alkylation of nAChR-SH. At 4 mM, d-TC raises $K_{30}$ values to $\sim 50 \mu M$ for MBTA, and $\sim 600 \mu M$ 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 increase in $K_{30}$ values for MBTA and BAC, respectively.

Provisionally, results of limited experiments (data not shown)
indicate that nicotinic pharmacology of the MBTA or BAC binding site is reflected in the rank order of inhibitory potencies of reversibly associating cholinergic ligands, which essentially parallel their ability to block toxin binding to reduced CNS nAChR (15). Nicotine and d-TC are the most potent inhibitors of MBTA or BAC labeling of toxin receptors, followed by decamethonium, hexamethonium, and, surprisingly, atropine. Carbachol, as well as ACh, are less potent. While concentrations of reversibly associating ligands necessary to block MBTA or BAC labeling of reduced CNS nAChR are markedly higher than those necessary to block toxin binding, such is also the case for interaction of reduced Torpedo nAChR with reversibly associating ligands, toxin, or affinity reagents (Mark McNamee, personal communication). Thus, the present results demonstrate that both reversibly associating nicotinic cholinergic agonists and antagonists retard specific reaction of MBTA or BAC with CNS nAChR.

Results of experiments examining α-Bgt competition toward specific reaction of MBTA or BAC with nAChR sites are summarized in Table II. Not surprisingly, they demonstrate that toxin prevents affinity reagent labeling of reduced toxin receptors.

Given the large excess of non-nAChR-SH over nAChR-SH available for reaction with cholinergic receptor affinity reagents, a series of experiments were conducted in an attempt to find conditions that would facilitate specific labeling of nAChR-SH with MBTA or BAC by reduction of non-specific reaction with non-nAChR-SH. When native nAChR is reacted with NEM, washed free of NEM, reduced with DTT, and alkylated with affinity reagent, K_{30} values are decreased, at most, 2-fold (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 in situ (33).

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 $\sim$ 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 nAChR-SH by reagent and NEM. Thus, the results reflect the larger rate constant for MBTA alkylation of nAChR-SH relative to that for BAC. Assuming that the rate constant for reaction of BAC with nAChR-SH is $\sim$ 5000 M$^{-1}$sec$^{-1}$ (29), the apparent rate constants for reaction with nAChR-SH are $\sim$ 500 M$^{-1}$sec$^{-1}$ for NEM and $\sim$ 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; 26) and 2.8$\cdot$10$^5$ M$^{-1}$sec$^{-1}$ for reaction of MBTA with peripheral nAChR (26). Thus, the results of the experiments reported here indicate reasonable agreement in relative rate constants for affinity alkylation of peripheral nAChR and CNS nAChR.

There has appeared a report that reaction of nAChR at frog neuromuscular junction and at the hatchetfish Mautner fiber-giant fiber synapse with sodium bisulfite, which presumably reacts with receptor
disulfide bonds through a heterocyclic cleavage mechanism, increases the physiological response to ACh (44). As with DTNB treatment of DTT-reduced CNS nAChR, treatment of native CNS nAChR with sodium bisulfite leaves receptor in a high-affinity state toward agonist (Lukas, unpublished observations). MBTA and BAC do not alkylate bisulfite-treated CNS nAChR with high affinity (Fig. 4.), indicating that the particular sulfhydryl(s) that reacts with MBTA or BAC is either sulfonated, or sterically blocked from alkylation with affinity reagent, following bisulfite treatment. However, as reported for peripheral nAChR (44), 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 nAChR are involved in oxidation-reduction state changes related to differences in affinity of nAChR for cholinergic agonists and reaction with affinity reagents. However, physiological (44) and the present biochemical evidence may be interpreted as demonstrating equivalence of the S-S loci involved in these events.
CONCLUSIONS

Following reduction of a receptor S-S bond with DTT, membrane-bound α-Bgt binding entities derived from rat brain interact specifically with affinity reagents designed to label well-characterized nAChR from the peripheral nervous system. Curaremimetic neurotoxin and reversibly associating nicotinic cholinergic agonists and antagonists retard the rate of affinity reagent reaction with nAChR-SH, presumably indicating that affinity reagents interact with nAChR near the cholinergic active site. Indications are that the relative rates of MBTA, BAC and NEM reaction with reduced α-BgtR are comparable to their relative rates of interaction with nAChR from the periphery. Furthermore, the MBTA/BAC site(s) on CNS α-BgtR respond to treatment of receptor with other S-S and SH-directed reagents in a manner strictly comparable to that seen for peripheral nAChR. The results may be taken as further biochemical evidence that CNS α-BgtR are authentic nAChR, sharing a remarkable number of properties with peripheral nervous system nAChR.

These results also suggest that a systematic physiological and biochemical study of toxin structure-function relationships is in order, with the purpose being to explain the existing controversy regarding antagonistic potency of curaremimetic neurotoxins at peripheral, central and autonomic cholinceptive loci.

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

The authors thank personnel in the Technical Information Division of the Lawrence Berkeley Laboratory for secretarial assistance, and express sincere gratitude to Dr. Mark G. McNamee, University of California at Davis, for supplying details of BAC synthesis prior to their publication, for his generous gifts of MBTA, and for valuable advice.
REFERENCES


16. Barrantes, F.J. Intrinsic fluorescence of the membrane bound


TABLE I

Determination of Membrane-Bound Sulfhydryl Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. of SH (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM---DTT</td>
<td>65</td>
</tr>
<tr>
<td>DTT</td>
<td>190</td>
</tr>
<tr>
<td>none</td>
<td>125</td>
</tr>
</tbody>
</table>

Free sulfhydryl groups in rat brain membranes were assayed by reaction with DTNB (42) in the presence of 300 μM sodium arsenite (43) to minimize reaction with residual DTT. 500 μl aliquots of membrane suspension were reacted with: a) 1.2 mM NEM, washed 3 times by dilution in 12 ml of Ringer's medium, centrifugation at 38000 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 38000 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 38000 g, supernatnats 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—DDT 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].
Membrane fractions are treated with DTT (0.3 mM, 20 minutes), and divided into 4 sets of 6 samples (200 μl aliquots). One set is treated with 0.3 mM NEM for 10 minutes, followed by exposure to affinity reagent (DTT-NEM). One set is exposed to buffer for 10 minutes, followed by exposure to affinity reagent (DTT). One set is exposed to 400 μM native α-Bgt for 10 minutes, followed by affinity reagent treatment (DTT-α-Bgt). One set is exposed to α-Bgt, only, without reaction with affinity reagent. Samples are diluted 60-fold, subjected to centrifugation at 40,000 g for 15 minutes, and resuspended in 5 ml buffer, sedimented and resuspended in 5 ml buffer again. After each overnight incubation, the sedimentation/resuspension process is repeated. On day six (138 hours) samples are assayed for specific [3H]-α-Bgt binding (30 minutes incubation, 4 tests, 2 blanks each set). DTT-NEM samples bind 16.0 ± 1.3 fmol [3H]-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 [3H]-α-Bgt (54% blocked) and DTT samples treated with BAC bind 3.8 ± 1.8 fmol [3H]-α-Bgt (76% blocked). Samples treated with native α-Bgt, only, bind 11.8 ± 2.3 fmol [3H]-α-Bgt (consistent

**TABLE II**

Inhibition of Affinity Labeling by α-Bgt

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Toxin Sites Blocked by 10 μM MBTA</th>
<th>Percent Toxin Sites Blocked by 10 μM BAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT-NEM a</td>
<td>0</td>
<td>0</td>
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<td>DTT a</td>
<td>54</td>
<td>76</td>
</tr>
<tr>
<td>DTT-α-Bgt b</td>
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<td>2</td>
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</tbody>
</table>

Membrane fractions are treated with DTT (0.3 mM, 20 minutes), and divided into 4 sets of 6 samples (200 μl aliquots). One set is treated with 0.3 mM NEM for 10 minutes, followed by exposure to affinity reagent (DTT-NEM). One set is exposed to buffer for 10 minutes, followed by exposure to affinity reagent (DTT). One set is exposed to 400 μM native α-Bgt for 10 minutes, followed by affinity reagent treatment (DTT-α-Bgt). One set is exposed to α-Bgt, only, without reaction with affinity reagent. Samples are diluted 60-fold, subjected to centrifugation at 40,000 g for 15 minutes, and resuspended in 5 ml buffer, sedimented and resuspended in 5 ml buffer again. After each overnight incubation, the sedimentation/resuspension process is repeated. On day six (138 hours) samples are assayed for specific [3H]-α-Bgt binding (30 minutes incubation, 4 tests, 2 blanks each set). DTT-NEM samples bind 16.0 ± 1.3 fmol [3H]-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 [3H]-α-Bgt (54% blocked) and DTT samples treated with BAC bind 3.8 ± 1.8 fmol [3H]-α-Bgt (76% blocked). Samples treated with native α-Bgt, only, bind 11.8 ± 2.3 fmol [3H]-α-Bgt (consistent

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with 72 hour 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. DTT-α-Bgt samples treated with MBTA and BAC bind $11.7 \pm 3.0$ fmol $[^3H]α$-Bgt (1% available toxin sites blocked by affinity reagent) and $11.6 \pm 1.9$ fmol $[^3H]α$-Bgt (2% available toxin sites blocked by affinity reagent), respectively.
FIGURE LEGENDS

Fig. 1. Blockade of $[^3H] \alpha\text{-Bgt}$ binding by reaction of brain membranes with cholinergic receptor affinity reagents. Levels of $[^3H] \alpha\text{-Bgt}$ specifically bound to membranes is plotted against the molar concentration (logarithmic scale) of MBTA (upper panel) or BAC (lower panel) present in the reaction mixture. Membranes were untreated ($\bullet$), treated with DTT (○), treated with DTT-NEM (○, lower panel), or treated with DTT-DTNB (○, upper panel), as described in EXPERIMENTAL PROCEDURE, prior to addition of affinity reagent. Also illustrated is the effect of DTT-NEM treatment on specific $[^3H] \alpha\text{-Bgt}$ binding (▲, upper panel). Data points represent single determinations, and, with the exception of the data for native membranes (see text), are typical of numerous replicate experiments.

Fig. 2. Modification of toxin binding rate by treatment of membranes with affinity reagent. Levels of specifically-bound $[^3H] \alpha\text{-Bgt}$ (fmol) are plotted as a function of time (minutes) 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 in EXPERIMENTAL PROCEDURE. Data points represent single determinations.
Fig. 3. Effects of reversibly associating cholinergic ligand on efficiency of affinity labeling. Levels of \(^{3}\text{H}\)-\(\alpha\)-Bgt bound (percent) are plotted as a function of affinity reagent concentration (logarithmic scale; molar) present in the reaction mixture. Membranes are treated with DTT and subjected to reaction with MBTA (upper panel) or BAC (lower panel) in the presence of 4 \(\mu\text{M}\) \(\alpha\)-tubocurarine (○) or 200 \(\text{mM}\) acetylcholine and 100 \(\mu\text{M}\) 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 seconds by dilution as otherwise described in EXPERIMENTAL PROCEDURE. Membranes were resuspended, diluted, and recentrifuged repeatedly over a 4 hour 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.

Fig. 4. Effect of sulfhydryl/disulfide group treatment on affinity labeling. Levels of \(^{3}\text{H}\)-\(\alpha\)-Bgt bound (percent) are plotted as a function of the molar concentration (logarithmic scale) of MBTA (upper panel) or BAC (lower panel). Membranes are treated with \(\text{Na}_2\text{S}_2\text{O}_5\) (○), \(\text{Na}_2\text{S}_2\text{O}_5\)-DTT (▲), or NEM-DTT (washed free of NEM prior to DTT reaction; ●), or native membranes are added to affinity reagent alone (▲) or affinity reagent plus 300 \(\mu\text{M}\)
NEM (●). Affinity reagent reaction is quenched, and samples subjected to toxin binding assay as described in EXPERIMENTAL PROCEDURE. Data points represent single determinations, but are representative of numerous replicate experiments.
Figure 2

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Figure 3
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Figure 4

[3H] α-Bgt bound (percent)

-Log [affinity reagent] (M)

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