glucocorticoid receptors, presumably via Schiff base formation with ε-amino groups of lysine residues. Following reduction with $NaBH_4$, which itself has no effect on receptor, a small 2.9S form of glucocorticoid receptor is produced.

We have observed that the 2.9S form of receptor obtained after pyridoxal phosphate treatment and $NaBH_4$ reduction has considerable stability in vitro. This should allow us to better analyze the properties of glucocorticoid receptors which until now have been difficult because of receptor instability.

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Effects of Thio-Group Modification and Ca^{2+} on Agonist-Specific State Transitions of a Central Nicotinic Acetylcholine Receptor†

Ronald J. Lukas,* Hiromi Morimoto, and Edward L. Bennett

ABSTRACT: Agonist-binding affinities of central nervous system nicotinic acetylcholine receptors (nAcChR) are sensitive to the duration of exposure to agonist. These agonist-induced changes in receptor state may be mimicked by appropriate modification of receptor thio groups and/or by manipulation of solvent ionic composition. In the absence of Ca^{2+}, the concentration of acetylcholine (AcCh) necessary to prevent half of specific ^3H-labeled α-bungarotoxin binding is ~1 mM for nAcChR treated with dithiothreitol (DTT) or DTT–*N*-ethylmaleimide (low-affinity states) and ~40 μM for nAcChR treated with DTT–5,5′-dithiobis(2-nitrobenzoic acid) or for native nAcChR pretreated with AcCh (high-affinity states). Addition of Ca^{2+} results in an increase in the effectiveness of AcCh toward blocking toxin binding. None of these treatments alters toxin or antagonist binding nor are there observed differences in Hill numbers for agonist binding. Agonists competitively inhibit toxin binding to low-affinity states, but noncompetitive inhibition is observed for binding to high-affinity states. Values of AcCh dissociation constants estimated from these data fall within the range of values determined physiologically with nAcChR from other systems. The data indicate that the redox state of brain nAcChR thio groups and Ca^{2+} may mediate physiologically important changes in the receptor state during activation and desensitization.

The selective response of neurotransmitter receptors to agonist is fundamental to their physiological role in regulation of nerve impulse initiation. Recently, reports have appeared describing agonist-specific changes in receptor affinity for cholinergic agonists. These changes have been detected by inhibition of curaremimetic neurotoxin binding to peripheral (Weiland et al., 1976, 1977; Weber et al., 1975; Colquhoun & Rang, 1976; Barrantes, 1976, 1978; Lee et al., 1977; Quast et al., 1978) and central [Lukas(iewicz) & Bennett, 1978a,b] nAcChR.[1,2] These results suggest that nAcChR selectively

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[1] Abbreviations used: nAcChR, nicotinic acetylcholine receptor(s); CNS, central nervous system; DTT, dithiothreitol; AcCh, acetylcholine; MalNEt, *N*-ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); α-Bgt, α-bungarotoxin; [^3H]-α-Bgt, ^3H-labeled α-bungarotoxin; EGTA, ethylene glycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; IC_{50}, concentration of competitor required to block 50% specific [^3H]-α-Bgt binding; K_D^{app}, apparent dissociation constant for [^3H]-α-Bgt binding; n_H, Hill number for competitor binding to nAChR.

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and actively responds to agonists and make possible speculation that the observed affinity states of nAcChR correlate with the different physiological states of the receptor. In these studies, it was shown that the low-affinity state of nAcChR is transient. That is, application of a cholinergic drug to nAcChR causes a time-dependent transformation of nAcChR to a high-affinity state. It is desirable to find conditions that would enable one to "freeze" the receptor in high- or low-affinity forms and thus more readily enable comparison of biochemical and physiological states of the receptor.

Toward elucidation of the molecular mechanisms underlying receptor function, chemical modification procedures and their consequences on the physiological properties of nAcChR have been instructive. Karlin & Bartels (1966) demonstrated that reduction of *Electrophorus electricus* electroplax with DTT inactivated the physiological response to AcCh. Application of MalNEt to DTT-reduced electroplax rendered this inactivation irreversible, but full response to AcCh was restored on treatment with Nbs_2. Thus, the involvement of receptor thio groups[3] in the biological activity of peripheral nervous system nAcChR was established.

Another fundamental property of receptor function is the selectivity displayed toward ions to be passed through the membrane in the postsynaptic current. It is important to determine whether these ions also play roles in regulating receptor function. They might act directly, by binding to specific sites on the receptor, or indirectly, e.g., by catalyzing chemical modification of the receptor. There is evidence that Ca^{2+} may participate in the natural desensitization of the receptor (Nastuk & Parsons, 1970) and that Ca^{2+} interacts with nAcChR [see Chang & Bock (1977) and references cited below].

On the foundation established by the elucidation of agonist-specific affinity alterations of the CNS nAcChR [Lukas(iewicz) & Bennett, 1978a,b], this report describes the effects of thio-group modification on the toxin-binding competition effectiveness of cholinergic drugs toward membrane-bound nAcChR derived from rat brain subcellular fractions. In addition, studies are described on the effects of ionic composition and cation specificity on affinity-state transitions of the CNS nAcChR.

Experimental Procedure

Methods for purification of α-Bgt from crude lyophilized venom of *Bungarus multicinctus* (Miami Serpentarium; Eterović et al., 1975a; Hanley et al., 1977) and for preparation of [^{3}H]-α-Bgt (sp act. 25 dpm/fmol, 95% bound by excess nAcChR from *Torpedo californica* electroplax) by catalytic reduction of iodinated α-Bgt under tritium gas [Eterović et al., 1975b; Lukas(iewicz) et al., 1978a] are as previously described.

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_2PO_4, pH 7.5, and 10 μM phenylmethanesulfonyl fluoride 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 2000g for 15 min. Supernatants are collected, by taking care to reject the white layer above the pellet. The pellet is resuspended in sucrose and centrifuged again. Supernatants are then pooled and subjected to centrifugation at 17800g for 15 min (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 $MgSO_4$, and 33 mM Tris, pH 7.4, with HCl) or, where indicated, in a Ca^{2+}-free Ringer's medium (1.8 mM $MgCl_2$ and 1–4 mM EGTA is substituted for $CaCl_2$), sedimented at 24000g for 15 min, and resuspended in Ca^{2+}-containing or Ca^{2+}-free Ringer's medium again. Samples are then either divided into aliquots for binding assays or subjected to treatment with thio-group reagents prior to binding assay as described below or in the figure and table legends. These preparations contain 15–25 fmol of toxin sites/mg of membrane protein. No further purification steps were followed as the yields of material are low and specific toxin binding activities are increased less than threefold on sucrose density gradients (Hanley et al., 1978) or by other selective sedimentation procedures (Morimoto, Lukas, and Bennett, unpublished experiments).

Thio-Group Modification. In general, membrane preparations at ~20 mg/mL are treated with 0.3 mM DTT for 20 min. Some DTT-treated membranes are further reacted with 0.3 mM Nbs_2 for 20 min or with 0.3 mM MalNEt for 5 min, followed by Nbs_2 reaction. In all cases, thio group reagent treated membranes are diluted in the appropriate Ringer's medium and subjected to centrifugation at 36000g 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 details for experiments using other reagents are described in the figure or table legends. Unless specifically noted, all manipulations are performed in Ca^{2+}-containing medium and at 0–4 °C.

Binding Assays. The extent of specific [^{3}H]-α-Bgt binding to membranes is determined as the difference in radioactivity bound to samples chased with 4 μM native toxin for 25 min following treatment with [^{3}H]-α-Bgt and blank samples treated with 4 μM native toxin for 30 min prior to the addition of [^{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. Following the native toxin chase period, membranes are subjected to two cycles of suspension in 3 mL of Ringer's medium and centrifugation for 15 min at 36000g. Supernatants are removed, and pellets are drained to dryness and then resuspended and quantitatively transferred to vials containing Aquasol-2 (New England Nuclear) for ^{3}H determination by liquid scintillation counting (Packard TriCarb 3375 or Beckman LS 9000; 30–40% efficiency). Typically, 500 cpm is specifically bound in the absence of cholinergic ligand out of 800–900 total cpm. All assays are carried out at 21 °C with shaking.

Affinities of cholinergic ligands for membrane-bound nAcChR are inferred from their ability to block toxin binding. For experiments using cholinergic ligands that are substrates for acetylcholinesterase, preincubation of membranes with 10 μM neostigmine or 100 μM eserine is sufficient to block enzymatic activity over the time course of the binding assay without affecting [^{3}H]-α-Bgt binding. Drug competition studies are carried out by one of two protocols. For preincubation experiments, cholinergic drug is added to membranes 30 min prior to the addition of [^{3}H]-α-Bgt. For coincubation experiments, effector and [^{3}H]-α-Bgt are simultaneously added

[2] CNS nAcChR are defined by specific interaction with α-Bgt (see Discussion).

[3] The term "thio groups" is used to designate organic disulfide and sulfhydryl residues.

to membrane samples. In preliminary experiments, the duration of exposure to [^{3}H]-α-Bgt at 10 nM was 30 min, in order to increase experimental sensitivity. For preincubation experiments, the results obtained from these single time point kinetic assays agree quantitatively with data on ligand effects on toxin binding derived from saturation isotherms or rate studies. This is also the case under conditions where receptor is "frozen" into one form or another by appropriate thio-group modification and/or variation in Ca^{2+} concentration. However, for cases where receptor is undergoing a time-dependent change from low- to high-affinity states, coincubation experiments using single time point assays yield only qualitative binding affinities. More accurate estimates of ligand affinity are determined from toxin binding rate or saturation experiments or from ligand binding to receptor frozen in low-affinity form. The Appendix should be consulted for a theoretical treatment of toxin binding and its modification in the presence of ligand.

Protein Concentration. Membrane proteins are determined by the method of Lowry et al. (1951).

Materials. Acetylcholine perchlorate was synthesized in our laboratory. Other chemicals and reagents are Tris, phenylmethanesulfonyl fluoride, carbachol chloride, decamethonium bromide, trimethylphenylammonium iodide, lobeline hydrochloride, β-mercaptoethanol, *p*-(chloromercuri)benzoate, MalNEt, and EGTA (Sigma); *d*-tubocurarine chloride, eserine, *S*-acetylthiocholine, butyrylthiocholine iodide, glutathione, neostigmine, and DTT (Calbiochem); tetraethylammonium chloride and nicotine hydrochloride (K & K Laboratories); hexamethonium chloride dihydrate (Schwartz/Mann); gallamine triethiodide (ICN); Nbs_2 (Aldrich); iodoacetamide (Nutritional Biochemical); and ascorbic acid (Baker).

Results

Thio-Group Modification and Ca^{2+} Effects. Levels of [^{3}H]-α-Bgt specifically bound to membrane-bound CNS nAcChR decrease as the concentrations of competing cholinergic ligands increase. An apparent affinity of nAcChR for ligand may be inferred from the concentration at which 50% of specific toxin binding is blocked (IC_{50}; see Appendix). The specific range of carbachol concentrations over which toxin binding is blocked varies depending on the duration of exposure of membranes to carbachol [Lukas(iewicz) & Bennett, 1978a,b] on reaction of membranes with thio group directed reagents and on the concentration of Ca^{2+} in the assay medium (Figure 1). IC_{50} values for carbachol competition toward toxin binding are $\sim$20 μM when native membranes are preexposed to carbachol in the presence of Ca^{2+}. IC_{50} values are also $\sim$20 μM for DTT–Nbs_2-treated membranes, where oxidation of sulfhydryl groups results and promotion of disulfide bond formation is presumably favored. Thus, CNS nAcChR is in a high-affinity state under these conditions. In contrast, IC_{50} values are increased when toxin and carbachol are simultaneously added to native membranes [Lukas(iewicz) & Bennett, 1978a,b] and are $\sim$200 μM for DTT–MalNEt-treated membranes, where sulfhydryl groups are irreversibly alkylated. Under these conditions, nAcChR is in a low-affinity state toward carbachol. IC_{50} values for carbachol are increased in the absence of Ca^{2+}. For example, preincubation assay in Ca^{2+}-free Ringer's medium yields IC_{50} values of $\sim$200 μM. These thio-group redox state and Ca^{2+} concentration dependent alterations in nAcChR binding affinity are observed only for cholinergic agonists. IC_{50} values are essentially the same (10 μM) for *d*-tubocurarine competition toward toxin binding to DTT–Nbs_2- or DTT–MalNEt-treated membranes and for assays in Ca^{2+}-free or Ca^{2+}-containing Ringer's medium (Figure 1). Thus, nAcChR exists in one state with respect to antagonist competition, irrespective of thio-group modifications or Ca^{2+} concentration.

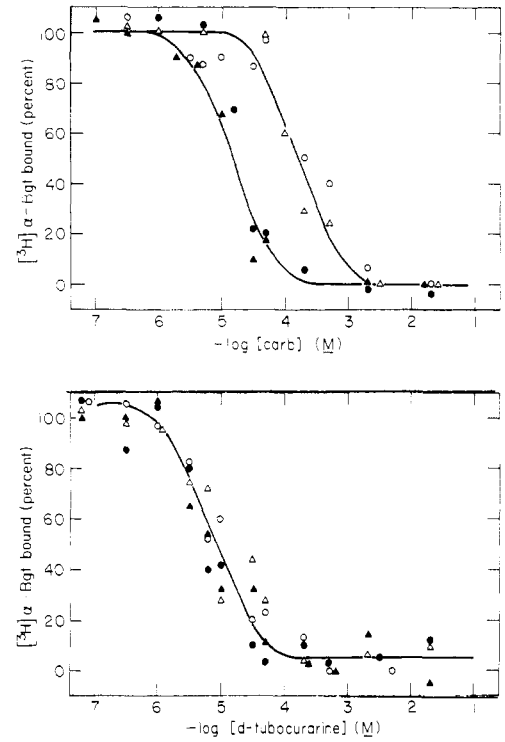

FIGURE 1: Cholinergic ligand competition for [^{3}H]-α-Bgt specific binding. Quantity of specific [^{3}H]-α-Bgt binding sites occupied by toxin (percent of maximum) is plotted against the molar concentration (logarithmic scale) of carbachol (upper) and *d*-tubocurarine (lower). DTT–Nbs_2-treated membranes (●); preincubation assay in Ringer's medium (▲); DTT–MalNEt-treated membranes (○); preincubation assay in Ca^{2+}-free Ringer's medium (△). Assays in 92 mM NaCl, 4 mM KCl, 1.4 mM $CaCl_2$ (or 1.4 mM $MgCl_2$ and 4 mM EGTA), 1.0 mM $MgSO_4$, 26 mM Tris, pH 7.4, and 1% bovine serum albumin, 250 μL final volume, 21 °C with shaking. Samples typically contain 3–4 mg of membrane protein. [^{3}H]-α-Bgt concentration is 10 nM and native toxin is at 4 μM at the termination of the assay. Identical results are obtained for DTT–Nbs_2- or DTT–MalNEt-treated membranes for co- and preincubation experiments. Data points represent single determinations, but plots are typical of numerous replicate experiments.

DTT treatment, which cleaves disulfide bonds and maintains reduced sulfhydryls, fails to alter the IC_{50} (10 μM) for *d*-tubocurarine competition (Figure 2). However, for carbachol, DTT treatment of membranes gives rise to a marked reduction in competition effectiveness toward toxin binding on coincubation (Figure 2). IC_{50} values are about 1 mM. A similar large reduction in toxin binding competition potency ($IC_{50} \simeq$ 2 mM) is observed for coincubation with AcCh, indicating that DTT treatment also yields nAcChR in a low-affinity state toward agonist. This effect is not a consequence of irreversible denaturation of nAcChR. The affinity of DTT-treated membranes for agonists is sensitive to preincubation with agonist. IC_{50} values are 50 μM when DTT-treated membranes are preincubated with AcCh prior to the addition of toxin (Figure 2). Furthermore, as indicated above, subsequent treatment with Nbs_2 completely reverses the effects of DTT.

IC_{50} values for a variety of cholinergic ligands, whose competition effectiveness toward toxin binding was ascertained under conditions as exemplified in Figures 1 and 2, are summarized in Table I. The effect of the presence of Ca^{2+} on agonist IC_{50} values is clearly shown in that the ratio of IC_{50} for assay in Ca^{2+}-free Ringer's medium to that in full Ringer's

Table I: IC_{50} Values (μM) for Competition vs. 10 nM [^{3}H]-α-Bgt[a]

drug	Rgr	Ca-free[b]	Nbs_2	MalNEt[c]	DTT[d]
acetylcholine	4	40 (10)	6	60 (10)	2000 (500)
S-acetylthiocholine	10	60 (6)	10	50 (5)	200 (20)
butyrylthiocholine	40	70 (2)	80	100 (1)	100 (3)
decamethonium	50	400 (8)	80	50 (0.5)	40 (1)
nicotine	1	10 (10)	3	30 (10)	10 (10)
carbachol	10	200 (20)	30	300 (10)	1000 (100)
trimethylphenylammonium	30	300 (10)	40	40 (1)	60 (2)
tetraethylammonium	80	800 (10)	80	800 (10)	5000 (60)
hexamethonium	1000	2000 (2)	1000	800 (0.8)	3000 (3)
gallamine	100	300 (3)	100	300 (3)	800 (8)
lobeline	20	200 (10)	30	60 (2)	60 (3)
d-tubocurarine	10	10 (1)	10	10 (1)	20 (2)

[a] Characteristic IC_{50} values are determined from inspection of data plotted as in Figure 1. Preincubation assays pertain to experiments done in full Ringer's medium (Rgr), in Ca^{2+}-free medium (Ca-free) and for DTT–MalNEt-treated membranes (MalNEt). Coincubation assays are done for DTT–Nbs_2-treated membranes (Nbs_2) and for DTT-treated membranes (DTT). DTT, Nbs_2, and MalNEt experiments are done in Ca^{2+}-containing Ringer's medium. [b] Values in parentheses denote ratios of IC_{50} values on preincubation in Ca^{2+}-free medium to those on preincubation in Ca^{2+}-containing Ringer's medium. [c] Values in parentheses denote ratios of IC_{50} values for inhibition of toxin binding to MalNEt-treated membranes to those for Nbs_2-treated membranes. [d] Values in parentheses denote ratios of IC_{50} values for DTT-treated membranes to those for preincubation assay in full Ringer's medium. IC_{50} values for preincubation in full Ringer's medium are 2000 μM for eserine and 400 μM for neostigmine. Note that IC_{50} values for DTT–Nbs_2- or for DTT–MalNEt-treated nAcChR are independent of the duration of preincubation time with ligand.

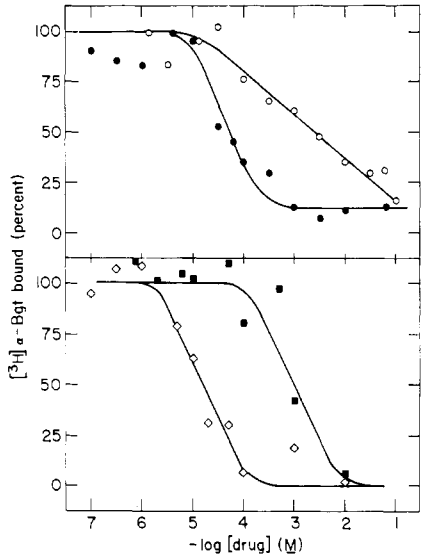

FIGURE 2: Effect of DTT treatment on ligand competition effectiveness. Membranes are treated with DTT as described under Experimental Procedure and washed free of DTT prior to initiation of the binding experiment. Percent specific [^{3}H]-α-Bgt sites occupied are plotted against the molar concentration of the competing ligand on a logarithmic scale for DTT-treated membranes. Preincubation assay in the presence of AcCh (●); coincubation assay in the presence of AcCh (○); coincubation assay with *d*-tubocurarine (◇); coincubation assay with carbachol (■). Assay conditions are as described in Figure 1 in the presence of Ca^{2+}. The apparent alteration in the Hill number in this experiment for AcCh competition on coincubation (○) reflects heterogeneity in receptor affinities for AcCh brought about by the transient nature of the low-affinity state. Data points represent single determinations, but plots are typical of numerous replicate experiments.

medium is ~10 for AcCh, *S*-acetylthiocholine, carbachol, and nicotine. In contrast, for antagonists, that ratio is ~1 for *d*-tubocurarine and ~3 for gallamine. The oxidation/alkylation state of membrane thio groups also selectively effects agonist IC_{50} values. The ratio of the IC_{50} value for DTT–MalNEt-treated membranes to that for DTT–Nbs_2-treated membranes is again ~10 for AcCh, *S*-acetylthiocholine, carbachol, and nicotine but is ~1 for *d*-tubocurarine and ~3 for gallamine. Further, the most marked decrease in ligand competition effectiveness toward toxin binding to DTT-treated membranes is observed for agonists. Ratios of IC_{50} for coincubation with DTT-treated membranes to IC_{50} for preincubation in Ringer's medium are in excess of 100 for both AcCh and carbachol and about 10 for *S*-acetylthiocholine and nicotine. The ratio for *d*-tubocurarine is ~2. Interestingly, the binding properties for some of the ligands tested do not fall into these empirical agonist or antagonist classifications, perhaps reflecting mixed agonist/antagonist potencies, local anesthetic properties, and changes in binding site accessibility affecting bulky ligands or suggesting that SH modification and Ca^{2+} effects are involved in different molecular processes (see below).

Table II: Preincubation Responsiveness to AcCh[a]

condition	IC_{50}, pre (μM)	IC_{50}, co (μM)
Ringer's medium	4	50 (12)
DTT, Ringer's medium	50	2000 (40)
DTT–Nbs_2, Ringer's medium	5	5 (1)
DTT–MalNEt, Ringer's medium	100	100 (1)
Ca^{2+}-free medium	40	100 (2)
DTT, Ca^{2+}-free medium	2000	2000 (1)

[a] Comparison of IC_{50} values for preincubation (pre) and coincubation (co) experiments for membranes treated as shown. [b] Values in parentheses denote ratios of IC_{50} values on coincubation to those on preincubation.

As shown by data summarized in Table II, native and DTT-treated nAcChR are responsive to pretreatment with AcCh when assays are done in the presence of Ca^{2+}. That is, IC_{50} values are lower for preincubation assay than for coincubation assay, reflecting increased affinity for agonist on pretreatment with agonist. In contrast, DTT-treated membranes are not responsive to AcCh pretreatment when assays are done in Ca^{2+}-free medium nor are DTT–Nbs_2- or DTT–MalNEt-treated membranes, irrespective of Ca^{2+} concentration. There is a small but significant change in AcCh competition effectiveness on preincubation in Ca^{2+}-free Ringer's medium.

Taken together, these results indicate that a high-affinity form (toward agonist) of nAcChR is frozen by DTT–Nbs_2 treatment. When assays are conducted in full Ringer's medium, the low-affinity states found on coincubation of native

Table III: Summary of Thio Group Directed Reagent and Ion Effects on AcCh IC_{50} Values

treatment[a]	IC_{50} (μM)
0.3 mM DTT	3000
1 mM β-mercaptoethanol	10
1 mM glutathione	5
1 mM ascorbate	4
1 mM *p*-(chloromercuri)benzoate	5
0.3 mM DTT-1 mM glutathione	100
0.3 mM DTT-1 mM ascorbate	30
0.3 mM DTT-1 mM *p*-(chloromercuri)benzoate	2000
0.3 mM DTT-0.3 mM MalNEt	80
0.3 mM DTT-1 mM iodoacetamide	60
0.3 mM DTT-0.3 mM Nbs_2	6
0.3 mM DTT-1 mM potassium ferricyanide	6
K^+-free medium	3
Na^+-free medium	2
Mg^{2+}-free medium	2

[a] Treatment is as follows. DTT, β-mercaptoethanol, glutathione, ascorbate, *p*-(chloromercuri)benzoate, Nbs_2, and potassium ferricyanide: 20 min, coincubation assay. MalNEt and iodoacetamide: 5 min, preincubation assay. For ion depletion experiments, preincubation assays were used. All experiments were performed in Ca^{2+}-containing medium.

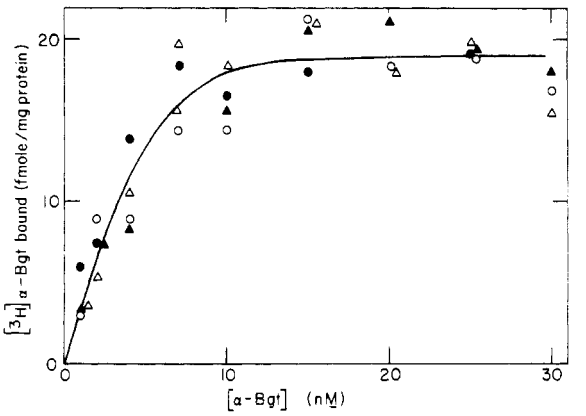

FIGURE 3: [^{3}H]-α-Bgt specific binding (fmol/mg of membrane protein) is plotted against toxin concentration (nM) for DTT–MalNEt-treated (○) and DTT–Nbs_2-treated (●) membranes and for assays conducted in Ca^{2+}-free (△) and Ca^{2+}-containing (▲) Ringer's media. Levels of specific toxin binding (fmol/mg of membrane protein) to a different membrane preparation treated with DTT at the concentrations indicated are 22.1, 0 mM; 19.2, 0.1 mM; 18.4, 0.3 mM; and 19.6, 1.0 mM. Assay conditions are as described in Figure 1, with data points from single determinations.

and DTT-treated membranes, respectively, are transient. That is, there is a time-dependent increase in affinity for agonist over the time course of the reaction. Nevertheless, a low-affinity state of nAcChR is preserved by DTT–MalNEt treatment or by DTT treatment when assays are conducted in the absence of Ca^{2+}. The increase in agonist affinity for native and DTT-treated nAcChR on preincubation with agonist is more marked in the presence of Ca^{2+}.

Specificity of Thio-Reagent, Cation and Agonist Effects. The results of experiments where a variety of thio group reactive compounds were tested for their effect on AcCh inhibition of toxin binding are summarized in Table III. DTT is the most potent agent tested toward increasing IC_{50} values (decreasing affinity) for AcCh. β-Mercaptoethanol, reduced glutathione, and ascorbic acid, which have less disulfide-directed activity than DTT (Cleland, 1964; Gorin et al., 1968), are less effective than DTT. Treatment with *p*-(chloromercuri)benzoate alone gives rise to IC_{50} values seen for preincubation assays in full Ringer's medium. In each case, prior treatment with DTT enhances the ability of these sulfhydryl group directed reagents to decrease the competition potency of AcCh. Alkylation of DTT-reduced membranes with iodoacetamide yields nAcChR that display IC_{50} values similar to that for DTT–MalNEt alkylated membranes. As for DTT–Nbs_2 treatment, oxidation of DTT-reduced membranes with potassium ferricyanide yields nAcChR with minimum IC_{50} values for AcCh competition. Thus, while sulfhydryl-directed reagents have limited ability to decrease AcCh competition IC_{50} values, their potency is increased when preceded by treatment with DTT, indicating that cleavage of disulfide bonds is important in manifestation of affinity-state changes. Alkylating agents and mild reducing agents irreversibly leave DTT-reduced nAcChR in a low-affinity state. Full diminution in AcCh affinity is seen only for treatment with DTT or DTT and *p*-(chloromercuri)benzoate. Oxidizing agents reverse DTT effects. Also summarized in Table III are data illustrating the absence of any affinity alteration effects in K^+-, Na^+-, or Mg^{2+}-free media and specificity of Ca^{2+} effects. More detailed data (not shown) indicate that 50% of toxin binding in the presence of 10^{-5} M AcCh is blocked at 600 μM Ca^{2+}. Toxin binding is blocked to 75% of maximum at 2 mM Ca^{2+} and completely blocked at ~20 mM.

These agonist-specific receptor state transitions are primarily attributable to effects on the affinity for reversibly-associating ligands (Figure 3). The maximum level of toxin binding in the absence of cholinergic effector and K_D^{app} values are largely unaffected, regardless of the thio-group treatment procedure and the presence or absence of Ca^{2+} in the assay medium.

Alterations in the Nature of Ligand Competition. The differences in apparent affinity (IC_{50}) of AcCh for thio group modified nAcChR and for assays conducted in Ca^{2+}-containing and Ca^{2+}-free media do not result in any striking alteration in the nature of AcCh competition for [^{3}H]-α-Bgt binding with respect to cooperative effects (Figure 4). Hill plots of AcCh competition data yield Hill numbers of about 1.0 for preincubation assays in full Ringer's medium and for assays using DTT–Nbs_2 treated membranes in Ca^{2+}-free or Ca^{2+}-containing buffer, indicating that there is no cooperativity in AcCh blockage of α-Bgt binding to nAcChR in a high-affinity state. While the Hill plot slope for preincubation assay in Ca^{2+}-free Ringer's medium is somewhat greater than 1.0 and $n_H \simeq 0.78$ for DTT–MalNEt-treated membranes, these values do not differ markedly from n_H for preincubation assays in full Ringer's medium.

The nature of AcCh inhibition of toxin binding is further elucidated by examination of its effects on toxin binding isotherms (Figure 5). Scatchard–Hofstee–Eadie plots of α-Bgt binding demonstrate that AcCh inhibition of toxin binding to DTT–Nbs_2-treated membranes or to native membranes on preincubation assay in Ca^{2+}-free Ringer's medium is noncompetitive. In these instances, as for preincubation assay with native membranes in full Ringer's medium [Lukas(iewicz) & Bennett, 1978b], the maximum number of available toxin sites is reduced, but K_D^{app} values are only somewhat altered (reflecting the increased observed potency of ligand toward blocking toxin binding at low concentrations, where the actual rate of toxin binding is slower). In contrast, AcCh inhibits toxin binding to DTT–MalNEt-treated membranes and DTT-treated membranes (on coincubation) in a simple competitive fashion, as previously shown for coincubation assays with native nAcChR in full Ringer's medium [Lukas(iewicz) & Bennett, 1978b]. That is, values of K_D^{app} are affected, but maximum binding levels are not altered in the presence of ACh. The advantage with DTT–MalNEt-treated and, to a lesser extent, DTT-treated mem-

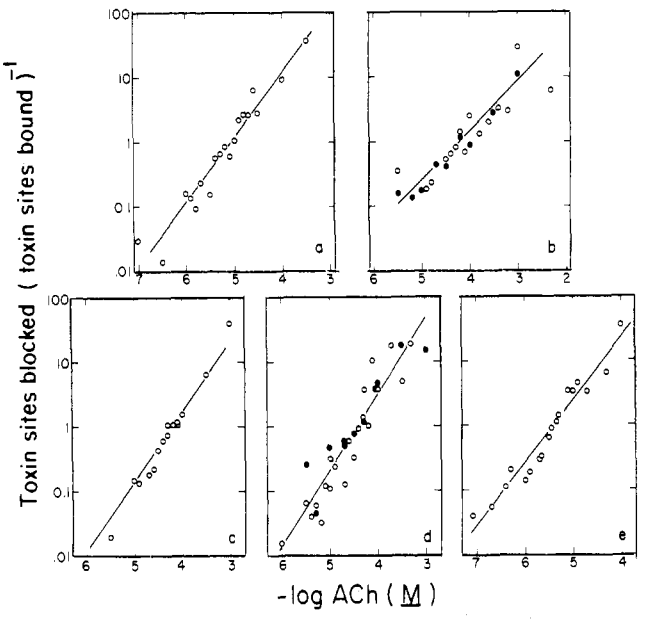

FIGURE 4: Hill plots of apparent AcCh binding as inferred from competition toward [^{3}H]-α-Bgt binding. Assays conducted in Ca^{2+}-free (Figure 4c,d) or Ca^{2+}-containing (Figure 4a,b,e) Ringer's medium. Hill numbers (±error slope determined from least-squares linear regression analysis) are (a) DTT-Nbs_2-treated membranes, 1.01 (±0.32); (b) DTT-MalNEt-treated membranes, 0.78 (±0.21); (c) DTT-Nbs_2-treated membranes, 1.09 (±0.24); (d) preincubation assay in Ca^{2+}-free Ringer's medium, 1.18 (±0.37); and (e) preincubation assay in full Ringer's medium, 0.95 (±0.32). Conditions are as described in Figure 1. Throughout, it is assumed that equilibrium between free ligand, free receptor, and ligand–receptor collision complexes is achieved before the first measurement of toxin–receptor complexes. Empirical support for this premise is derived from congruence of data for coincubation and preincubation conditions when receptor is frozen in high- or low-affinity forms by DTT-Nbs_2 or DTT-MalNEt treatment, respectively. Equilibration of high-affinity ligand–receptor complexes with ligand–receptor collision complexes is achieved within the 30-min preconditioning period for preincubation conditions [Lukas(iewicz) & Bennett, 1978b]. Determination of Hill coefficients for ligand binding is appropriate only under equilibrium conditions for ligand binding, and this criterion is met by the conditions of the displayed assays. Data points represent the average of duplicate measurements. Different symbols denote data from different experiments.

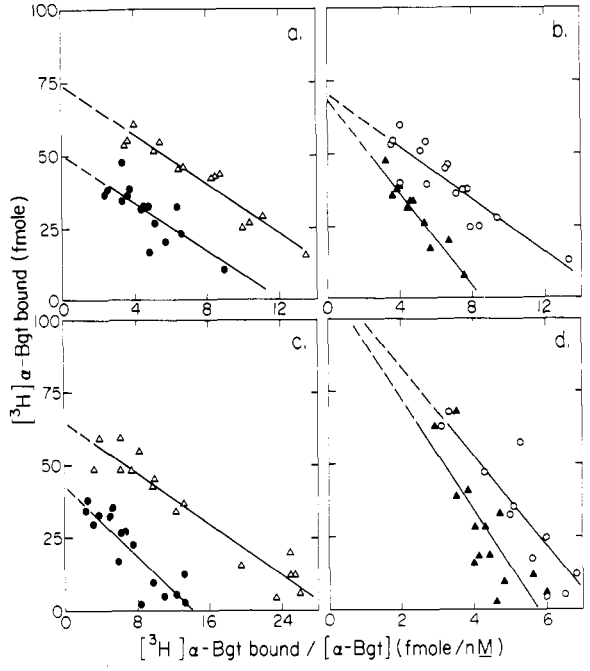

FIGURE 5: Mechanism of AcCh inhibition of [^{3}H]-α-Bgt binding. Hofstee–Eadie–Scatchard plots of specific toxin sites occupied vs. (sites occupied)(toxin concentration)$^{-1}$ yield values for V_{max} and slope respectively (±standard error as determined from least-squares linear regression analysis of the data) for (a) DTT-Nbs_2-treated membranes in the presence of 0 μM AcCh, 74 ± 3, −4.2 ± 0.4 (△) and 4 μM AcCh, 50 ± 5, −4.2 ± 1.0 (●); (b) DTT-MalNEt-treated membranes in the presence of 0 μM AcCh, 70 ± 5, −4.5 ± 0.7 (○) and 40 μM AcCh, 70 ± 4, −8 ± 0.8 (▲); (c) preincubation assay in Ca^{2+}-free medium in the presence of 0 μM AcCh, 65 ± 3, −2.2 ± 0.2 (△) and 40 μM AcCh, 43 ± 4, −3.0 ± 0.5 (●); and (d) coincubation assay with DTT-treated membranes in the presence of 0 μM AcCh, 113 ± 14, −15 ± 3 (○) and 300 μM AcCh, 118 ± 21, −19 ± 5 (▲). Assay conditions are as described in Figure 1 or 2 for membrane protein concentrations of 12.8 (a), 12.4 (b), 11.8 (c), and 18.3 mg/mL (d). Duration of exposure to [^{3}H]-α-Bgt is 60 min. Data points represent single determinations.

branes is that the resultant low-affinity states are more stable relative to those for native membranes.

For the different receptor-state manipulations used, the initial rate of toxin binding is also affected differently by AcCh. In the absence of AcCh, the linear dependence of toxin binding rate data, shown on a semilogarithmic transform in Figure 6, yields virtually identical slopes for assays in the absence of Ca^{2+} and for DTT-, DTT-Nbs_2-, and DTT-MalNEt-treated membranes. Thus, the overall rate constant for toxin binding is unaffected by Ca^{2+} concentration and thio-group manipulation in the absence of ligand. However, when AcCh is present, rates are decreased in every case, but with different AcCh concentration dependencies as cited above and in different manners. For DTT-Nbs_2-treated membranes and for preincubation assays in Ca^{2+}-free [or full Ringer's medium; see Lukas(iewicz) & Bennett (1978b)], the maximum ordinate value is approached asymptotically in the displayed data, which is calculated by assuming that the maximum number of toxin sites is unaltered in the presence of AcCh. However, if the data are replotted by using an experimentally derived, reduced number of available toxin binding sites (specific binding after 60-min incubation in the presence of AcCh at the concentration indicated), they are found to superimpose with the data taken in the absence of AcCh (not shown), yielding an identical rate constant for toxin binding as the control experiments. Thus, in these cases, the overall rate constant for toxin binding to available sites is unaltered, but the number of sites is depressed in the presence of AcCh. As is the case for coincubation assays with native membranes [Lukas(iewicz) & Bennett, 1978b], data on semilogarithmic toxin binding rate transforms plateau abruptly for coincubation assays with DTT-treated membranes, illustrating the transient nature of the low-affinity state detected under these conditions. There is a progressive increase in affinity for AcCh as incubation times increase, so that while the initial rate of toxin binding is not markedly affected, at longer times, further toxin binding is prevented. In contrast to the above cases, for DTT-MalNEt-treated samples, the data in Figure 6 are linear. Thus, the number of available toxin binding sites is unaltered, but the rate constant for binding to those sites is diminished, as ascertained from the shallower slope.

The results of toxin binding rate and saturation isotherm studies may be summarized as follows. For nAcChR in high-affinity states, that is, for preincubation in Ca^{2+}-free or Ca^{2+}-containing Ringer's medium or for DTT-Nbs_2-treated membranes, AcCh has the predominant effect of noncompetitively reducing the number of available toxin sites without having primary effects on K_D^{app} values or on the rate constant for toxin binding to available sites. The diminished affinity of AcCh for native and DTT-treated membranes on coincubation assay is transient. However, the major effect of AcCh in these cases is only to slow down the toxin binding rate and

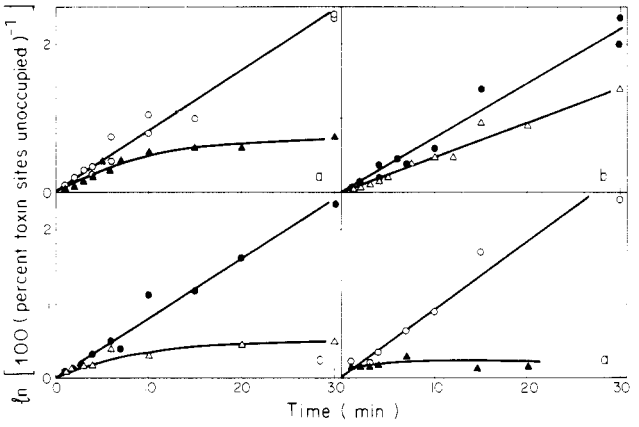

FIGURE 6: Effect of AcCh on rate of toxin binding. The rate equation for interaction of toxin with receptor may be written $kt = ([T_0] - [R_0])^{-1} \ln.[[R_0]([T] - [RT^*])[T_0]^{-1}([R_0] - [RT^*])^{-1}]$ where $[R_0]$ is the total receptor concentration (maximum number of toxin sites in the absence of cholinergic effector), $[T_0]$ is the total toxin concentration, k is the binding rate constant, and $[RT^*]$ is the quantity of toxin–receptor complexes at the time of duration of exposure to [^{3}H]-α-Bgt, t. For $[T_0] \gg [R_0]$ and $[RT^*]$, the equation reduces to $kt = [T_0]^{-1} \ln [[R]_0([R_0] - [RT^*])^{-1}] = [T_0]^{-1} \ln [100 \text{ (percent toxin sites unoccupied)}^{-1}]$. Data are given for the rate of toxin binding in the presence of 10 nM [^{3}H]-α-Bgt, yielding $k \simeq 8 \times 10^6$ min mol for all preparations. (a) DTT–Nbs_2-treated membranes in the presence of 0 μM AcCh (○) and 3 μM AcCh (▲); (b) DTT–MalNEt-treated membranes in the presence of 0 μM AcCh (●) and 40 μM AcCh, $k \simeq 4.8 \times 10^6$ min mol (△); (c) preincubation assay in Ca^{2+}-free medium with 0 μM AcCh (●) and 40 μM AcCh (△); and (d) coincubation assay with DTT-treated membranes in the presence of 0 μM AcCh (○) and 2 mM AcCh (▲). Assays are as described in Figure 1 or 2 except that the duration of exposure is for the time indicated on abscissa. Data points represent the average of duplicate determinations, and linear plots are fit to least-squares linear aggression analysis of the data.

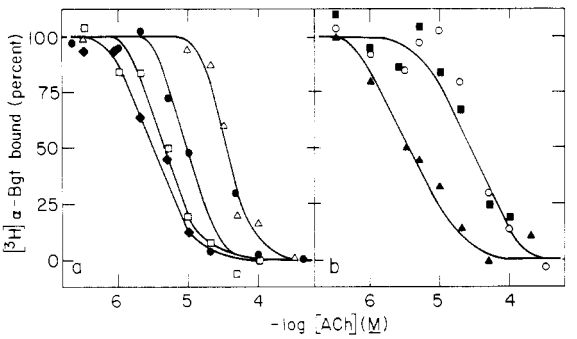

FIGURE 7: Rate of affinity-state transformation as a function of duration of exposure to or removal of Ca^{2+}. (a) Coincubation assay is initiated by using membranes prepared in the presence of 1 mM EGTA (△) or using EGTA-treated membranes after addition of Ca^{2+} (4 mM final total concentration) for 0 (●), 1 (□), and 30 min (♦) prior to addition of AcCh and toxin. (b) Preincubation assay is conducted by using membranes prepared in Ca^{2+}-containing Ringer's medium (▲), or reaction is initiated by addition of toxin to membranes exposed to AcCh for 30 min after exposure to EGTA (4 mM final concentration) for 0 (○) and 30 min (■). Data points represent single determinations.

increase K_D^{app} values without altering the quantity of available toxin sites. These low-affinity states may be preserved by alkylation of reduced thio groups with MalNEt. In no case is there observed cooperativity in agonist blockage of toxin binding to receptor.

Time Course of State Changes. An experiment conducted to estimate the time course of affinity-state transition on addition or depletion of Ca^{2+} is shown in Figure 7. On addition of Ca^{2+}, the rate of transformation to a high-affinity state is on the order of minutes, similar to that seen for the rate of transition when the duration of preincubation with

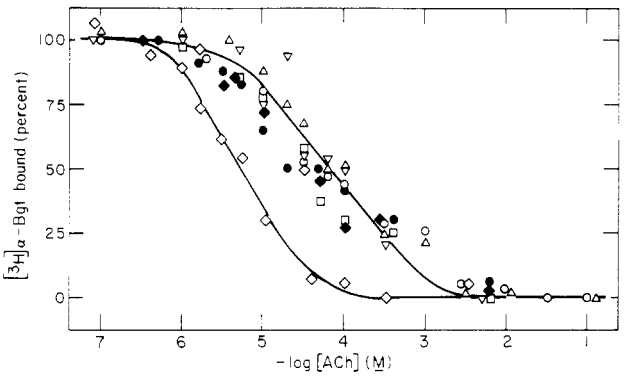

FIGURE 8: Effect of cholinergic ligand on alkylation of membrane-bound nAcChR. Effectiveness of AcCh toward blockage of α-Bgt binding for DTT–Nbs_2-treated (◇) and DTT–MalNEt-treated membranes (○), for DTT-treated membranes exposed to ligand at listed concentrations, and for times indicated prior to addition of MalNEt. (△) 3 mM trimethylphenylammonium, 1–5 min; (□) 1 mM lobeline, 1 min; (▽) 0.1–1 mM *d*-tubocurarine, 1–5 min; (♦) 1 mM decamethonium, 1 min; (●) 1 mM carbachol, 1 min. Exposure to carbachol for longer times (30 min) prior to addition of MalNEt had no blocking effect. Assay conditions are as described in Figure 1. In all cases, ligand is *not* removed from membranes by dilution and centrifugation prior to addition of MalNEt. All MalNEt treatments are for 5 min and are followed by dilution and centrifugation sequence before addition of Nbs_2. Three cycles of dilution and centrifugation, with exposure to Nbs_2 throughout, are used to reduce free-ligand concentrations to levels that do not alter toxin binding before final resuspension in full Ringer's medium for binding assay.

AcCh in full Ringer's medium is increased [Lukas(iewicz) & Bennett, 1978b]. Reversal of the increase in AcCh affinity, as determined from an experiment where depletion of Ca^{2+} is effected by addition of EGTA, is much faster, as even on addition of EGTA simultaneous with initiation of the toxin binding assay, IC_{50} values are about equal to those for AcCh competition for toxin binding to membranes treated with EGTA throughout. Thus, the time course of increase in nAcChR affinity for AcCh as mediated by Ca^{2+} (or by preincubation with AcCh) is slower than reversal of that process.

Ligand–Thio Group Interactions. A variety of cholinergic ligands were used in attempts to prevent MalNEt alkylation of DTT-reduced nAcChR, i.e., permit the increase in affinity for AcCh on subsequent treatment with Nbs_2. The results show no ability of either agonist or antagonist to protect from alkylation (Figure 8).

Sequence Effects. In order to test the possibility that nAcChR thio-group modification and Ca^{2+} play sequential roles in receptor activation and desensitization, respectively, experiments were conducted testing temporal effects of thio-group modification and exposure to or removal of Ca^{2+}. It is evident (Table IV) that the absence or presence of Ca^{2+} during thio-group modification, for the most part, has no effect on those manipulations; DTT–Nbs_2- and DTT–MalNEt-treated membranes exhibit similar IC_{50} values when assays are conducted in a given Ringer's medium. The one possible exception is reaction in Ca^{2+}-free medium of DTT–MalNEt-treated membranes, which seem to be refractory to subsequent addition of Ca^{2+} on assay. Solvent effects are therefore primarily attributable to the absence or presence of Ca^{2+} in the assay medium. For assays conducted in Ca^{2+}-free medium, IC_{50} values for AcCh competition toward α-Bgt binding are about 10-fold higher than for assays conducted in the presence of Ca^{2+}. This result indicates that neither sulfhydryl modification nor Ca^{2+} alone exclusively controls the affinity states of nAcChR. Rather, the effects of Ca^{2+}

Table IV: Combined Solvent and Thio-Group Effects

reaction buffer	treatment procedure: thio treatment	assay medium	IC_{50} (μM)
Ringer	MalNEt-Nbs_2	Ringer	100
	Nbs_2		4
	MalNEt-Nbs_2	Ca^{2+} free	3000
	Nbs_2		40
Ca^{2+} free	MalNEt-Nbs_2	Ringer	2000
	Nbs_2		5
	MalNEt-Nbs_2	Ca^{2+} free	2000
	Nbs_2		40

[a] Crude mitochondrial fractions are suspended in Ca^{2+}-containing Ringer's medium (Ringer) or Ca^{2+}-free medium (Ca^{2+} free) and subjected to 20-min exposure to 0.3 mM DTT, followed by centrifugation and resuspension in the appropriate reaction buffer. Treatment was with Nbs_2 or MalNEt, followed by Nbs_2, as described under Experimental Procedure, in the appropriate reaction buffer. Final pellets, freed of excess thio-group reagent, are resuspended in the indicated assay medium for coincubation assay (Nbs_2) or preincubation assay (MalNEt) under conditions as described in Figure 1.

and thio-group manipulation appear to be additive.

Discussion

From the results of toxin binding inhibition studies, it has been demonstrated that the state of CNS α-Bgt binding entities (which increasingly exhibit the properties of and may be identified as CNS nAcChR), with respect to affinity for cholinergic agonists, is sensitive to manipulation of (presumably receptor) thio groups and to the presence of Ca^{2+}. CNS nAcChR in a high-affinity form can be produced by preincubation with AcCh or by oxidation of DTT-reduced nAcChR by Nbs_2. A permanent low-affinity state of nAcChR results from treatment of DTT-reduced membranes with MalNEt, and transient low-affinity states may be detected in coincubation experiments by using DTT-reduced and native membranes. Affinities for agonist in each case are further diminished in the absence of Ca^{2+}; Ca^{2+} and thio-group effects appear to be additive. [^{3}H]-α-Bgt binding to high-affinity state(s) is inhibited noncompetitively by AcCh (AcCh inhibition of toxin binding is *not* overcome by increased toxin concentration or by lengthening the duration of exposure to toxin), but inhibition of toxin binding to low-affinity state(s) is competitive. Neither agonists nor antagonists protect nAcChR from alkylation by MalNEt. None of these treatments alters toxin binding properties to membranes in the absence of AcCh nor are there marked alterations in cooperativity of agonist inhibition of toxin binding.

Relevance of α-Bgt Binding Sites. These results, taken together with previous observations on the pharmacology (Schmidt, 1977; Moore & Brady, 1976; McQuarrie et al., 1976; Speth et al., 1977), physical properties (Lowy et al., 1976; Moore & Brady, 1977; Salvaterra & Mahler, 1976), subcellular localization (de Blas & Mahler, 1978; Salvaterra et al., 1975; Eterović & Bennett, 1974; Tindall et al., 1978), regional distribution (Morley et al., 1977; Segal et al., 1978; Speth et al., 1977), and histological disposition (Silver & Billiar, 1976; Polz-Tejera et al., 1975; Lentz & Chester, 1977; Vogel et al., 1977; Hunt & Schmidt, 1978) of CNS α-Bgt receptors, provide strong evidence for their identity as CNS nAcChR. Recent demonstrations of postsynaptic, toxin-mediated antagonism of physiological CNS cholinergic responses (Freeman, 1977; Nistri & Arensen, 1978; Miledi & Szczepaniak, 1975) and accumulating evidence that toxin antagonistic potency in parasympathetic ganglia is structure dependent (Chiappinelli & Zigmond, 1978; Ravdin et al., 1978) point to ultimate reconciliation of previous physiological results (Miledi & Szczepaniak, 1975; Duggan et al., 1976) with the biochemical and ultrastructural evidence that supports identity of CNS curaremimetic toxin binding sites as authentic nAcChR.

In this connection, the noncompetitive nature of agonist competition for α-Bgt binding to nAcChR in high-affinity states has several implications. First, the results show that agonist binding to a site other than the α-Bgt binding site is more effective at noncompetitively blocking toxin binding than is agonist competitive inhibition of toxin binding to nAcChR in low-affinity states. This supports our initial suggestion [Lukas(iewicz) & Bennett, 1978b] that there may be structural, and perhaps functional, heterogeneity in agonist binding sites on CNS nAcChR; that is, there may be toxin-insensitive, agonist binding sites that control ion translocation. The results also support our conclusion that the apparent heterogeneity in agonist binding sites is mirrored in differences in α-Bgt and *Dendroaspis viridis* toxin 4.7.3 recognition by CNS nAcChR (Hanley et al., 1978). The precise relationship between these biochemical hints of binding-site heterogeneity and the potency or impotency of toxin-mediated antagonism is yet to be elucidated.

Thio-Group Involvement in Receptor Function. Despite the fact that this study deals with CNS nAcChR and utilizes a toxin binding competition experimental design, parallels may be drawn between this work and results of other groups using different techniques to determine agonist affinities for peripheral nervous systems nAcChR. Since the original description by Karlin & Bartels (1966) of alteration in the physiological response of *E. electricus* electroplax nAcChR to cholinergic agonist, there have appeared reports detailing similar thio group reagent effects on agonist affinity by using direct (Eldefrawi & Eldefrawi, 1972; Schiebler et al., 1977) and indirect (Miller et al., 1979) binding assays. Thio-group modification also perturbs in vivo (Mittag & Tormay, 1970; Rang & Ritter, 1971; del Castillo et al., 1972; Ben-Haim et al., 1973; Lindstrom et al., 1973) and in vitro (Kasai & Changeux, 1971; Schiebler et al., 1977) responses of chemically excitable nAcChR to cholinergic agonists. Reasoning that receptor-state changes are related to physiological function, we may conclude that CNS nAcChR contain thio groups that may participate in the specific responses of nAcChR to agonist that lead to receptor activation and/or desensitization.

The detailed involvement of CNS nAcChR thio groups in those responses, however, apparently differs from that for thio groups on peripheral nAcChR. For example, DTT treatment potentiates the response of eel electroplax nAcChR to hexamethonium and decamethonium, but DTT–MalNEt alkylation abolishes the response (Karlin & Bartels, 1966; Karlin, 1969). Our results show that the affinities of hexamethonium and decamethonium for native, reduced, and alkylated CNS nAcChR are about the same and that both ligands actually bind with slightly higher affinity to alkylated nAcChR than to DTT–Nbs_2-treated membranes. By the assumption that in vitro binding affinities are related to in vivo activation potencies, these data indicate that peripheral and CNS nAcChR may be distinguished. Most of the evidence in this report suggests that disulfide bonds maintain CNS nAcChR in a high-affinity state. Sulfhydryl-group reagents, particularly *p*-(chloromercuri)benzoate, reduce agonist-mediated responses of electroplax nAcChR (Karlin & Bartels, 1966) but are considerably less potent toward altering the

affinity state of CNS receptor, suggesting another peripheral CNS nAcChR difference. Further, there exists evidence that agonist treatment causes diminution of sulfhydryl-group reactivity in *T. californica* electroplax nAcChR (Suarez-Isla & Hucho, 1977) and that a thio group is near the active site of peripheral receptors and serves as a site for affinity labeling of peripheral receptors (Karlin & Winnik, 1968; Froehner et al., 1977). However, our attempts to block MalNEt alkylation of CNS nAcChR with cholinergic agonists and antagonists failed. It is of interest to determine whether CNS nAcChR may be further distinguished, as is frog neuromuscular junction nAcChR (Lindstrom et al., 1973), from other peripheral nAcChR by differences in reactivity toward a nAcChR thio-group affinity reagent, such as [4-(*N*-maleimido)-benzyl]trimethylammonium. Lastly, there has recently emerged evidence for disulfide bond controlled dimerization of peripheral nAcChR components (Chang & Bock, 1977; Hucho et al., 1978; Hamilton et al., 1977; Witzemann & Raftery, 1978), but changes in receptor affinity for agonist as a function of receptor dimerization have not been detected [Schiebler et al. (1977) and Gibson et al. (1976); however, see Chang & Bock (1977)]. While we cannot address this issue directly using unpurified, membrane-bound nAcChR, the results summarized on Tables I, III, and IV, indicating differences in ligand affinity for nAcChR treated with DTT, DTT-alkylating agents, and DTT-reducing agent, may suggest a complex involvement of disulfide and sulfhydryl group(s) at different sites on CNS nAcChR.

Ca^{2+} Involvement in Receptor Function. Further parallels may be drawn between the results reported here and those of other investigators using other experimental systems and procedures regarding effects of Ca^{2+} on receptor function. In the presence of Ca^{2+} the affinity of cholinergic agonists for membrane-bound nAcChR from *Torpedo marmorata* electroplax is increased (Cohen et al., 1974). There is also an acceleration of the usual time course of decrease in amplitude of the efflux of Na^+ ions from excitable vesicles seen on exposure to carbachol (Sugiyama et al., 1976), an in vitro model of desensitization. Interestingly, there is other evidence that Ca^{2+} and cholinergic agonists may displace one another from binding sites on electroplax nAcChR (Chang & Newmann, 1976; Eldefrawi et al., 1975; Rubmassen et al., 1976; Mihovilovic et al., 1977). The Ca^{2+} concentration where all of these effects, and the effects described in this paper, are observed are similar ($\sim$1 mM). Evidence exists suggesting that Ca^{2+} may accelerate, if not induce, nAcChR desensitization in vivo (Manthey, 1966; Bartels, 1971; Nastuk & Parsons, 1970; Magazaniak & Vyskocil, 1970; Schnitzler et al., 1975; Devore & Nastuk, 1977). It has been suggested that accumulation of Ca^{2+} intracellularly may cause nAcChR desensitization (Nastuk & Parsons, 1970), although interaction of Ca^{2+} with any hidden sites exposed concomitantly with receptor activation may yield the same effect. Our data support the contention that Ca^{2+} alters the nAcChR affinity for agonist in a manner consistent with its postulated role in desensitization, i.e., by inducing a receptor state with high affinity for agonists. An intriguing possibility that might explain all the Ca^{2+}-effect observations is that Ca^{2+}-mediated desensitization is accompanied by a net release of AcCh from other (low-affinity) binding sites.

General Scheme for Receptor Function. One might further draw on the results in order to relate the observed affinity states of CNS nAcChR to physiological states of the receptor.

A pertinent implication of the present results is that, since desensitized nAcChR is thought to be refractory to α-Bgt binding (Lester, 1972; Miledi & Potter, 1975), the high-affinity state may correspond to the desensitized state of the CNS nAcChR. From the relation $K_I^{app} = IC_{50}\,(1 + T/K_D^{app})^{-1}$, where T = concentration of toxin ($\simeq$10 nM), $K_D^{app} \simeq 2$ nM, and K_I^{app} is an apparent inhibition constant for cholinergic ligand and an approximation of the ligand–nAcChR dissociation constant, one calculates a K_I^{app} for AcCh binding to the high-affinity state of $\sim$1 μM. The K_I^{app} for AcCh binding to MalNEt-alkylated nAcChR is $\sim$10 μM and for binding to the transient low-affinity state found on coincubation assay with DTT-treated membranes is $\sim$300 μM. While direct correlation of these data with AcCh concentrations necessary to activate nAcChR in vivo is difficult, the K_I^{app} values fall within the range of effective physiological AcCh concentrations for activation of native receptor (Lester et al., 1975, 1978; Katz & Miledi, 1973; Dionne & Stevens, 1975; Hartzell et al., 1975; Fertuck & Salpeter, 1976).

It is attractive to speculate, based on the reasonable agreement between in vivo and in vitro results and on the work of Karlin (1969) and Nastuk (1977) and their respective co-workers, that changes in specific receptor thio-group oxidation state are manifest upon receptor activation and that Ca^{2+} may somehow be involved in receptor desensitization. A provisional scheme may be written

$$A + R \text{ (low affinity)} \xrightarrow{\text{SH modification}} AR^* \xrightarrow{Ca^{2+}} AD \text{ (high affinity)} \quad (1)$$

where A is cholinergic agonist, R is nAcChR in the at-rest configuration, R* is activated nAcChR, and D is desensitized nAcChR. One might predict that, in the absence of Ca^{2+}, DDT-reduced, DDT–MalNEt-alkylated, and DTT–Nbs_2-oxidized nAcChR would have identical affinities for AcCh as reduced and/or alkylated nAcChR in the presence of Ca^{2+}, with AcCh binding with higher affinity to DTT–Nbs_2-treated nAcChR in the presence of Ca^{2+}. Not all of these expectations are borne out, as Ca^{2+} and thio-group effects appear to be additive for DTT–MalNEt- and DTT–Nbs_2-treated membranes and for preincubation assays with DTT-treated nAcChR. However, the data are consistent with a simple model as shown in eq 1, if R* has an intermediate affinity for AcCh and if exposure to Ca^{2+} potentiates AcCh binding to *all* states of nAcChR, including the physiologically important conversion of R* (oxidized nAcChR) to D. [It is interesting to note that there has appeared a report suggesting that some SH oxidizing agents can mimic the ability of AcCh to alter postsynaptic potentials at cholinoceptive junctions (Sobrino & del Castillo, 1972).] Thus, a simple, but not unique, model might accommodate the present data and the implications of Karlin's (1969) and Nastuk's (1977) discoveries.

Regardless of the precise mechanism by which CNS nAcChR is activated and desensitized, it is clear that thio-group modification and Ca^{2+} effects are involved in affinity-state changes of nAcChR. The exact relationship between the different agonist affinity states of CNS nAcChR detected in these studies and the physiologically identifiable at-rest, activated, and desensitized receptor remains unclear but is subject to elucidation by carefully planned in vivo and in vitro studies of a homogeneous population of nAcChR.

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Appendix

Application of Michaelis–Menten Treatment to Toxin Binding. Let the reaction of toxin with receptor in the absence of ligand be described by

$$R + T \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RT \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RT^* \tag{2}$$

for rate constant, k_i, where R represents free receptor, T is free toxin, and RT and RT* represent reversible toxin–receptor collision complexes and experimentally detectable, slowly reversible, toxin–receptor complexes, respectively. Under equilibrium conditions, the concentrations of R, RT, and RT* remain constant, as do the ratios R/RT/RT*. When this condition is reached, simple Michaelis–Menten type treatments of the data are permissible (Goldstein et al., 1969; Goodman & Gilman, 1975). The concentration of RT* at equilibrium is given by

$$[RT^*] = \frac{V[T]}{\bar{K} + [T]} \tag{3}$$

where $V = [R_0]k_3(k_3 + k_4)^{-1}$ and $\bar{K} = k_2k_4k_1^{-1}(k_3 + k_4)^{-1}$.

A special condition exists when k_3 and k_4 are small and a true equilibrium condition is not reached. This will occur for the short incubation times, t, used in most of the studies described above. Since k_4 is low [$t_{1/2}$ for decay of toxin–receptor complexes is ~70 h; Lukas(iewicz) & Bennett, 1978a] for short t, it can be assumed to equal zero. Further, assume that equilibrium between T, R, and RT is achieved rapidly. Over the time course and at the toxin concentrations used in our pre-steady-state reactions (30 min at 10 nM or less [^{3}H]-α-Bgt), the rate of formation of RT* is essentially first order in T. This situation holds only if the ratio of R/RT is constant and the rate of RT* formation equals the rate of depletion of R + RT. Expressed mathematically (see Aldridge & Reiner, 1972)

$$v = \frac{d[RT^*]}{dt} = -\frac{d[R]}{dt} - \frac{d[RT]}{dt} \tag{4}$$

Since

$$-\frac{d[R]}{dt} = k_1[R][T] - k_2[RT] \tag{5}$$

and

$$-\frac{d[RT]}{dt} = k_3[RT] + k_2[RT] - k_1[R][T] \tag{5a}$$

$$v = \frac{d[RT^*]}{dt} = k_3[RT] \tag{6}$$

and the equilibrium between R and RT yields

$$k_1[R][T] = (k_2 + k_3)[RT] \tag{7}$$

From conservation of receptor

$$[R_0] = [R] + [RT] + [RT^*] \tag{8}$$

Substituting from eq 7

$$([R_0] - [RT^*]) = \left(\frac{k_2 + k_3}{[T]k_1} + 1\right)[RT] \tag{9}$$

and

$$v = \frac{d[RT^*]}{dt} = k_3[RT] = \frac{k_3([R_0] - [RT^*])[T]}{\frac{k_2 + k_3}{k_1} + [T]} \tag{10}$$

which takes the form $v = V'[T](\bar{K}' + [T])^{-1}$ where the precise definitions for $V' = k_3([R_0] - [RT^*])$ and $\bar{K}' = (k_2 + k_3)k_1^{-1}$ are somewhat different than in the true equilibrium condition. Nevertheless, Michaelis–Menten transforms, including Scatchard–Hofstee–Eadie plots, may be used to evaluate V' and $\bar{K}'$.

In the presence of competing ligand, there exists an equilibrium between free ligand, L, free receptor, and ligand–receptor collision complexes, RL, with characteristic dissociation constant, K_L, defined by

$$R + L \underset{K_L}{\rightleftharpoons} RL \tag{11}$$

as

$$K_L = \frac{[R][L]}{[RL]} \tag{12}$$

Equation 8 must be modified to reflect the existence of RL

$$[R_0] = [R] + [RT] + [RL] + [RT^*] \tag{13}$$

Since, from eq 7 and 12

$$\frac{[RT]k_2 + k_3}{k_1[T]} = [R] = \frac{K_L[RL]}{[L]} \tag{14}$$

$$([R_0] - [RT^*]) = \left(\frac{k_2 + k_3}{k_1[T]} + 1 + \frac{(k_2 + k_3)[L]}{k_1[T]K_L}\right)[RT] \tag{15}$$

whence

$$v = \frac{d[RT^*]}{dt} = \frac{k_3([R_0] - [RT^*])[T]}{\frac{k_2 + k_3}{k_1} + [T] + \frac{(k_2 + k_3)[L]}{k_1K_L}} \tag{16}$$

Equation 16 has the form

$$v = \frac{V'[T]}{\bar{K}' + [T] + \frac{\bar{K}'[L]}{K_L}} \tag{17}$$

for V' and $\bar{K}'$ as defined above, which is analogous to equations for Michaelis–Menten competitive inhibition—in this case, of ligand toward toxin binding.

Furthermore, the integrated form of eq 16 gives the effect of ligand on the apparent first-order rate constant by

$$\frac{d[RT^*]}{([R_0] - [RT^*])} = \frac{k_3[T]dt}{\frac{k_2 + k_3}{k_1} + [T] + \frac{(k_2 + k_3)}{k_1}\frac{[L]}{K_L}} \tag{18}$$

and

$$\ln \frac{[R_0]}{([R_0] - [RT^*]_t)} = \frac{k_3t[T]}{\frac{k_2 + k_3}{k_1} + [T] + \frac{(k_2 + k_3)}{k_1}\frac{[L]}{K_L}} \tag{19}$$

For [L] = 0, the observed rate constant is

$$k_{obsd} = \frac{k_3[T]}{\bar{K}' + [T]} \tag{20}$$

and for finite [L]

$$k_{obsd} = \frac{k_3[T]}{\bar{K}' + [T] + \frac{\bar{K}'[L]}{K_L}} \tag{21}$$

Toxin Binding with Simultaneous Desensitization. The effect of ligand-induced alterations in receptor state to the exclusion of toxin binding may be predicted from the following.

Let k_D be the rate constant for transformation of RL to RL′, a receptor state incapable of binding toxin, by

$$R + L \rightleftharpoons RL \overset{k_D}{\rightleftharpoons} RL' \quad (22)$$

The conservation of receptor expression must be expanded accordingly

$$[R_0] = [R] + [RT] + [RL] + [RT^*] + [RL'] \quad (23)$$

and an equation is derived for the rate of formation of RT*

$$\frac{d[RT^*]}{dt} = \frac{k_3([R_0] - [RL'] - [RT^*])[T]}{\bar{K}' + [T] + \frac{\bar{K}'[L]}{K_L}} \quad (24)$$

In integrated form

$$\ln \frac{([R_0] - [RL'])}{([R_0] - [RL'] - [RT^*])} = \frac{kt[T]}{\bar{K}' + [T] + \frac{\bar{K}'[L]}{K_L}} \quad (25)$$

Consequently, the effect of such a process is to diminish the maximum number of sites available for interaction with toxin, without, if $[L]\bar{K}'(K_L)^{-1}[T]^{-1} < \bar{K}'[T]^{-1} + 1$, altering the apparent rate constant for toxin binding to available sites. That is, the ligand will behave as a noncompetitive inhibitor of toxin binding. At any time t, the number of available toxin binding sites is defined by

$$R_0(1 - e^{-\lambda_T[T]t} - e^{-\lambda_L[L]t}) \quad (26)$$

where λ_T and λ_L are the overall rate constants for formation of RT* and RL′, respectively.

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Regulation of Glycolipid Biosynthesis: Effects of Virus Infection and Drug-Induced Translational Inhibition on Glycolipid Metabolism†

Robert Anderson

ABSTRACT: Suppression of HeLa cell protein synthesis by either viral infection or translation-inhibiting drugs induces alterations of cell glycolipid concentrations such that there is accumulation of the simple glycolipid species (mono- and diglycosylceramides) as well as a depletion of the more complex ones (triglycosylceramides and gangliosides). In addition, the cellular pool of free ceramides is increased two- to threefold over that found in control cells. The in vitro activities of UDP-glucose:ceramide glucosyltransferase and UDP-galactose:glucosylceramide galactosyltransferase in homogenates prepared from streptovitacin A treated HeLa cells were found to decline progressively with increasing times of drug treatment when exogenous ceramide or glucosylceramide was utilized as carbohydrate acceptor. However, if endogenous ceramide was used as glucose acceptor, the activity of UDP-glucose:ceramide glucosyltransferase in homogenates from cells pretreated for 6 h with translational inhibitor was approximately twofold higher than that found in control cell homogenates, presumably as a result of the increased ceramide concentration in drug-treated cells. The source of increased ceramide in such cells is uncertain but does not appear to be derived from an impairment of ceramide incorporation into sphingomyelin. The results suggest that one component of cellular control of glycolipid biosynthesis may be the regulation of free ceramide levels.

In a previous report from this laboratory (Anderson & Dales, 1978), it was shown that infection of HeLa or L cells with vaccinia virus produced quantitative changes in the cellular glycolipid compositions, in that those of simple, neutral glycolipids, particularly ceramide monohexoside (CMH[1]),

† From the Department of Microbiology and Immunology, University of Western Ontario, London, Ontario N6A 5C1, Canada. *Received November 27, 1978; revised manuscript received February 21, 1979.* This research was supported by a grant from the Medical Research Council of Canada.

[1] Abbreviations used: CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; gangliosides G_{M2}, *N*-acetylgalactosaminyl(sialyl)galactosylglucosylceramide; G_{M3}, sialylgalactosylglucosylceramide; G_{D3}, sialylsialylgalactosylglucosylceramide; UDP-Gal, uridine diphosphogalactose; UDP-Glu, uridine diphosphoglucose; FCS, fetal calf serum; ME medium, minimal essential medium; PBS, phosphate-buffered saline; VSV, vesicular stomatitis virus.

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