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Allosteric Effects of Volatile Anesthetics on the Membrane-Bound Acetylcholine Receptor Protein

II. Alteration of α-Bungarotoxin Binding Kinetics

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SUMMARY

The acetylcholine receptor (AcChR) protein isolated from *Torpedo californica* possesses two binding sites for α-bungarotoxin. Although chemical modification and ligand-binding experiments have suggested that the two toxin binding sites are dissimilar, the toxin associates to its two sites on AcChR at identical rates. Incubation of AcChR-enriched membranes with volatile anesthetics and aliphatic alcohols retards the rate of association of toxin to one of its two binding sites on the high-affinity conformer of the protein. Kinetic plots of toxin binding characteristic of a single bimolecular association to AcChR become biphasic after incubation with these organic perturbants. Therefore, anesthetics and aliphatic alcohols alter the tertiary structure of the high-affinity conformer as well as stabilizing it relative to the low-affinity conformer. The differential response of the two toxin binding sites to these organic perturbants provides additional evidence of the non-equivalence of the binding loci.

INTRODUCTION

Volatile anesthetics and aliphatic alcohols alter the conformational state of the membrane-bound AcChR[1] protein by preferentially stabilizing a high-affinity form for agonists (1). In terms of the concerted model for allosteric transitions (2), these organic ligands serve as positive heterotropic effectors of the high-affinity conformer. In this report, we wish to describe an additional feature of the conformational perturbation of the AcChR from *Torpedo californica* by volatile anesthetics and aliphatic alcohols. In particular, we wish to demonstrate that anesthetics differentially affect the structure of the two toxin binding sites in the high-affinity conformer, causing an alteration in their kinetic and thermodynamic properties. Since the two sites can be distinguished in the presence of anesthetic, our studies provide further evidence for their non-equivalence in the membrane-associated AcChR of *T. californica* (3, 4).

EXPERIMENTAL PROCEDURE

Materials and methods have been summarized in the preceding report (1).

RESULTS

Toxin binding in absence of anesthetics. The rates of toxin binding in the absence of anesthetic exhibit second-order kinetics (5). When the toxin concentration is in excess, the rate of the formation of the receptor-toxin complex can be completely characterized by a single exponential for several half-lives. The second-order rate constant for the formation of the receptor-toxin complex calculated from the data in Fig. 1 is 1.94×10^5 M^{-1} sec^{-1} and 1.86×10^5 M^{-1} sec^{-1} for *lines 1* and *2*, respectively. In general, for different preparations of AcChR and [^{125}I]α-Bgt, second-order rates of $1.3–2.0 \times 10^5$ M^{-1} sec^{-1} are obtained. If a reversible complex between AcChR and toxin forms prior to the irreversible step, the dissociation constant for this complex must be sufficiently higher than the toxin concentration used in these experiments (0.3–0.4 μM). In general, no deviation from second-order kinetics is observed in the toxin concentration range from 0.1 to 0.5 μM, in agreement with published reports (6).

Lines 1 and *2* of Fig. 1 were obtained with the same stock of [^{125}I]α-Bgt solution 1 day and 34 days after iodination, respectively. The good agreement of the bimolecular rate constants indicates that the preparation of toxin was stable over that period of time. Occasionally, biphasic toxin binding curves were obtained with toxin preparations 1 or 2 months old. Aged toxin preparations which exhibited distinctly biphasic rates of toxin binding were never used in the studies described below. The

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[1] The abbreviations used are: AcChR, acetylcholine receptor; α-Bgt, α-bungarotoxin; carb, carbamylcholine; MBTA, 4-(*N*-maleimido)-benzyltrimethylammonium iodide.

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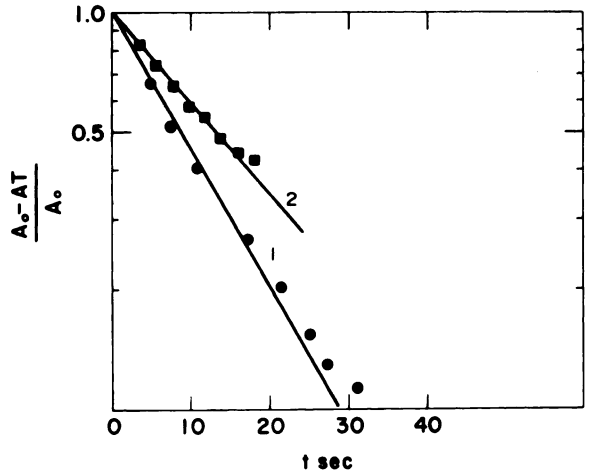


FIG. 1. *Association kinetics of [^{125}I]α-Bgt with Torpedo membranes*

The fraction of uncomplexed toxin binding sites on the membrane, $(A_0 - AT)/A_0$, is plotted versus time t after initiation of the toxin-binding reaction on a semilog scale. Reaction mixtures were 0.045 μM in toxin binding sites and 0.42 μM in [^{125}I]α-Bgt (●) or 0.040 μM in toxin binding sites and 0.28 μM in [^{125}I]α-Bgt (■).

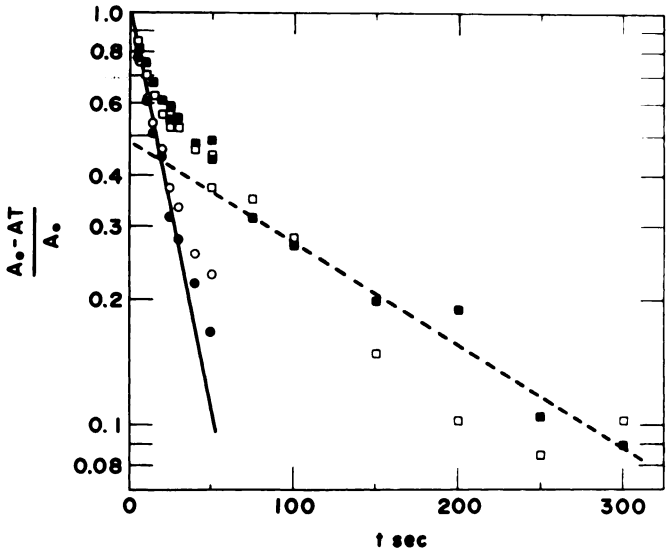


FIG. 2. *Association kinetics of [^{125}I]α-Bgt with membrane-bound AcChR after pretreatment with chloroform*

Membranes (0.044 μM in toxin binding sites) were pretreated for 30 min in the absence of anesthetic (●) or pretreated under an atmosphere of 1% (○), 3% (■), or 6% (□) chloroform followed by addition to [^{125}I]α-Bgt (0.42 μM) and assayed for the entire time course of toxin binding. —— is a least-squares fit of the data obtained for the first two half-lives of the reaction in the absence of chloroform; - - - is a least-squares fit of the data obtained after treatment of the membranes under 3% chloroform for reaction times of 75 sec or longer.

stability of the toxin was markedly enhanced by the inclusion of bovine serum albumin, 1 mg/ml.

The bimolecular rate constant for toxin binding depends on temperature and ionic conditions. In the range between 6.5° and 29°, Arrhenius plots are linear and the activation energy for the association of toxin with the AcChR is 16.5 kcal/mole. Divalent cations inhibit the toxin-binding reaction in the millimolar range (7). For example, we find that substitution of 1 mM EDTA with 1 mM $CaCl_2$ inhibits the reaction approximately 2-fold. A sodium ion concentration of 0.1 M was used for all experiments.

Biphasic plots in presence of anesthetic and aliphatic alcohols. In contrast to the data reported in Fig. 1, plots of natural logarithm A/A_0 versus t obtained with [^{125}I]α-Bgt in excess were distinctly biphasic after pretreatment of membranes for 30 min at 21° in the presence of 3% and 6% chloroform (Fig. 2). The membrane-bound AcChR exhibits a highly cooperative response to chloroform concentration. Although the kinetics of toxin binding can adequately be described by a single exponential for several half-lives for membranes incubated with up to 1% chloroform, the transition to a biphasic kinetic profile is complete by 3% chloroform and remains unchanged up to 6% chloroform. Since the transition from the low-affinity form to the high-affinity form of the AcChR is complete after incubation under 3% chloroform (1), the biphasic kinetics of toxin binding shown in Fig. 2 do not reflect this transition. Moreover, the identity of the kinetic behavior at 3% and 6% chloroform indicates that the nonlinear kinetics are not due to the preferential binding of chloroform at one of the two toxin binding sites. The effect of 3% chloroform on these toxin-binding kinetics could be reversed by passage of air over anesthetic-treated samples for 30 min prior to the onset of the toxin-binding assay.

Halothane also causes the toxin-binding kinetics to become biphasic (Fig. 3A). The concentrations of halothane required to produce this effect are somewhat higher than those required to convert the AcChR to a high-affinity state. Ethanol and butanol, in the concentration ranges required for conversion to the high-affinity state, also cause the appearance of biphasic curves of toxin binding. Octanol at concentrations of 1–2 mM, its limits of solubility, does not cause the rate of toxin binding to become biphasic even though octanol is an effective ligand for the conversion to the high-affinity form of the AcChR at these concentrations (Fig. 3B). The reason for this anomalous behavior of octanol is unclear. However, it is interesting to note that chloroform, halothane, butanol, and ethanol are capable of prolonging the duration of miniature end-plate currents observed at the neuromuscular junction under certain conditions (8–10) and also produce biphasic kinetics of toxin binding. Octanol shortens the duration of miniature end-plate currents (11) and does not produce biphasic toxin-binding kinetics.

Kinetic analysis of biphasic plots. The kinetic constants for the biphasic plots of toxin binding in the presence of perturbants were calculated using the general expression (12, 13)

$$A/A_0 = \alpha e^{-k_f t} + (1 - \alpha)e^{-k_s t} \quad (1)$$

where A_0 is the total concentration of toxin binding sites, A is the concentration of unbound AcChR at time, t; k_f is the pseudo-first order rate constant for the fast-binding phase; and α and $1 - \alpha$ represent the fraction of the reaction governed, respectively, by k_f and k_s. If $k_f \gg k_s$,

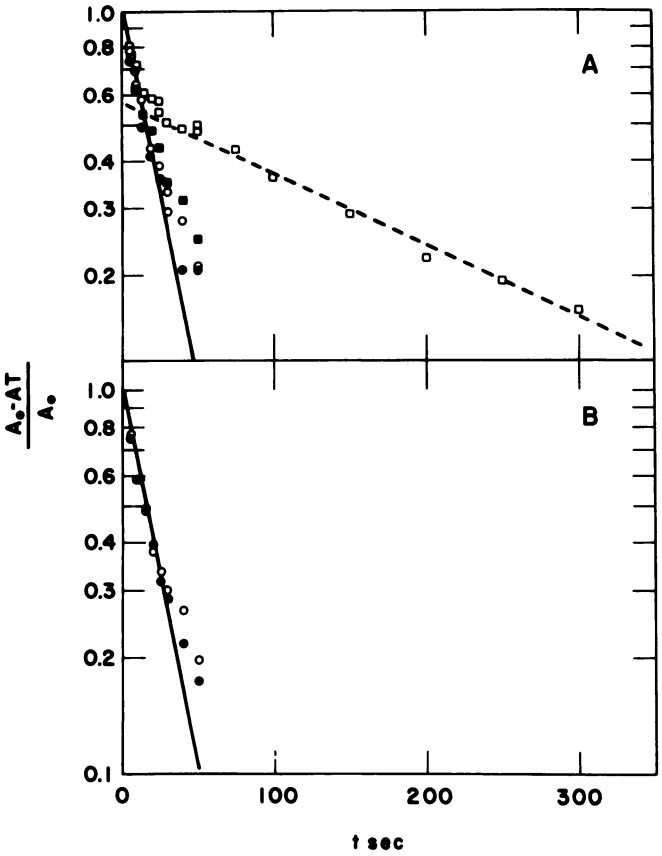


FIG. 3. *Association kinetics of [^{125}I]α-Bgt with the membrane-bound AcChR after pretreatment with halothane or octanol*

Membranes were pretreated for 30 min under an atmosphere of 3% (●), 5% (○), 8% (■), or 15% (□) halothane (*A*); or pretreated for 30 min with 1.25 mM (●) or 2.5 mM (○) octanol (*B*) prior to initiation of the toxin-binding reaction. Concentrations of toxin binding sites and [^{125}I]α-Bgt are the same as those in Fig. 2. —— in *A* and *B* is identical with that in Fig. 2, and - - - in *A* is obtained from the data with 15% halothane in a fashion analogous to that of - - - in Fig. 2.

Eq. 2 is valid when $t \gg 1/k_f$:

$$A/A_0 \approx (1 - \alpha)e^{-k_s t} \qquad (2)$$

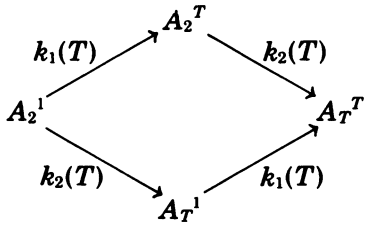
According to this expression, the slope of ln A/A_0 versus t at long times will have an intercept of $1 - \alpha$ at $t = 0$ and a slope of $-k_s$. Subtraction of the slow phase from A/A_0 at the short times after initiation of the reaction yields Eq. 3:

$$A/A_0 - (1 - \alpha)e^{-k_s t} = z = \alpha e^{-k_f t} \qquad (3)$$

Accordingly, plots of ln z versus t should be linear with an intercept of α and a slope of $-k_f$.

The rates constants for binding of toxin in the presence of the perturbants noted above are reported in Table 1. The most significant correlation emerging from these data is that the biphasic behavior results from the preferential inhibition of binding at one of the two toxin sites. The slow phase of toxin binding observed in the presence of perturbants is roughly 5 to 20-fold less rapid than the pseudo-first order rate constant observed in the absence of any perturbant. In contrast, the rapid phase of the reaction in the presence of all perturbants only ranges up to 2-fold faster than the control.

Possible mechanisms. There are two distinct kinetic mechanisms which would be consistent with the observed biphasic rates of toxin binding. In the first case, the perturbant could create two classes of toxin binding sites prior to the addition of the toxin. These two classes would have different intrinsic rates for the binding of the toxin to the AcChR (Scheme I). The rate constants k_f and k_s of Eq. 1 could be directly associated with the product of toxin concentration and k_1 and k_2, respectively. The upper pathway would clearly represent the predominant one to the double-occupied AcChR.



SCHEME I

In the second mechanism, the perturbant would affect subunit-subunit interactions and induce negative cooperativity between the two sites. In this model, the AcChR binds one molecule of toxin at essentially the control rate. However, the kinetic and thermodynamic properties of the site at which the second toxin molecule interacts

TABLE 1

Kinetic analysis of biphasic curves of toxin binding obtained in the presence of volatile anesthetics and primary alcohols

Membranes (0.044 μM in toxin binding sites) were pretreated for 30 min with the indicated concentration of perturbant prior to addition of [^{125}I]α-Bgt (0.42 μM) and assayed for the entire time course of toxin binding.

Perturbant	k_{app} sec^{-1a}	k_{fast} sec^{-1b}	k_{slow} sec^{-1c}	k_{fast}/k_{slow}	Intercept[d]
None	4.68×10^{-2}	—	—	—	—
Chloroform					
1%	4.33×10^{-2}	—	—	—	—
3%	2.72×10^{-2}	$4..88 \times 10^{-2}$	5.61×10^{-3}	8.7	0.48
6%	3.01×10^{-2}	5.92×10^{-2}	6.01×10^{-3}	9.8	0.45
Halothane					
3%	4.47×10^{-2}	—	—	—	—
5%	3.96×10^{-2}	—	—	—	—
8%	3.96×10^{-2}	—	—	—	—
15%	3.15×10^{-2}	9.24×10^{-2}	4.31×10^{-3}	21.4	0.57
Octanol					
1.25 mM	4.78×10^{-2}	—	—	—	—
2.50 mM	4.68×10^{-2}	—	—	—	—
Butanol					
0.1 M	3.85×10^{-2}	9.36×10^{-2}	9.28×10^{-3}	10.1	0.51
Ethanol					
0.5 M	4.62×10^{-2}	—	—	—	—
2.0 M	2.77×10^{-2}	5.33×10^{-2}	1.93×10^{-3}	27.6	0.53

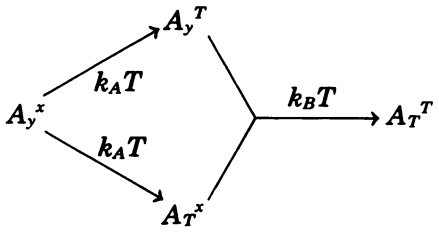
[a] Determined from least-squares analysis of the first 40% of the reaction.

[b] Determined from least-squares analysis of the data when toxin-binding rates were clearly biphasic in accordance with Eq. 3.

[c] Determined from least-squares analysis of the data when toxin-binding rates were clearly biphastic in accordance with Eq. 2.

[d] Determined from the intercept obtained from least-squares analysis of the slow phase of toxin binding in accordance with Eq. 2 when toxin binding rates were clearly biphasic.

are altered by the binding of the first toxin molecule in the presence of anesthetics and aliphatic alcohols. The mechanism can be summarized by Scheme II:



SCHEME II

where binding to either of these sites occurs with a microscopic pseudo-first order rate constant of k_A yielding A_y^T or A_T^x. Once a site is occupied, binding to the second site occurs with a slower pseudo-first order rate of k_B. The explicit expression in this case is summarized by Eq. 4.

$$A/A_0 = \frac{(k_A - k_B)}{(2k_A - k_B)} e^{-2k_A t} + \frac{k_A}{(2k_A - k_B)} e^{-k_B t} \quad (4)$$

The parameters of Eq. 1 can be defined by direct comparison with Eq. 4, which has the same general form. If $k_A \gg k_B$, the pre-exponential factor (α of Eq. 1) should approach 0.5, the same value anticipated from Scheme I, which presumes intrinsic heterogeneity. Since values of α of 0.5 were obtained (Table 1), artifactual heterogeneity in the AcChR preparation is unlikely to be responsible for the biphasic kinetics of toxin binding. However, this value of α cannot distinguish between Scheme I and Scheme II.

Heterogeneity in the toxin induced by anesthetics can be excluded as an explanation for the biphasic curves by carrying out the kinetic measurements in the presence of receptor excess. Under these conditions, the bimolecular rate constant is 1.19×10^5 M^{-1} sec^{-1} following incubation with 6% chloroform. This value closely corresponds to the bimolecular rate for the fast phase of toxin binding (1.3×10^5 M^{-1} sec^{-1}) in the presence of 6% chloroform under the conditions of toxin excess (Table 1). The most important result, localizing the perturbing effect to the receptor and not the toxin, is that the reaction with receptor excess can be analyzed as a single first-order reaction where the experimental pseudo-first order rate constant represents the product of the second-order rate constant and the AcChR concentration.

Affinity of the two sites for carb in the presence of anesthetic. It was of interest to determine whether the binding sites ligated in the rapid phase and those bound in the slow phase had similar affinities for carb. This was investigated by measuring rates of toxin binding in the presence of 0.1 M butanol after pretreatment with various concentrations of carb. Under these conditions, all of the AcChR should be in a high-affinity state and the biphasic curves generated should reflect the two classes of toxin binding sites. Each phase of the reaction could be analyzed, and a dissociation constant for carb calculated from the inhibition of toxin binding. Using 0.044 μM AcChR and 0.42 μM toxin, the kinetics of toxin binding were measured after preincubation with 0.1 M butanol and 0.5 μM carb. Least-squares analysis of the data (Fig. 4) shows that the slow phase of the reaction (1000 sec and longer) has an amplitude of 0.5 and a rate constant of 7.83×10^{-4} sec^{-1}. The fast phase of the reaction exhibits a rate constant of 3.31×10^{-3} sec^{-1}. From these data and the rates for the two phases of the reaction in 0.1 M butanol in the absence of carb (Table 1), the dissociation constants for carb to the sites which bind toxin rapidly and slowly are 0.018 μM and 0.046 μM, respectively. In a similar experiment using 0.25 μM carb, the corresponding dissociation constants were 0.018 μM and 0.049 μM, respectively.

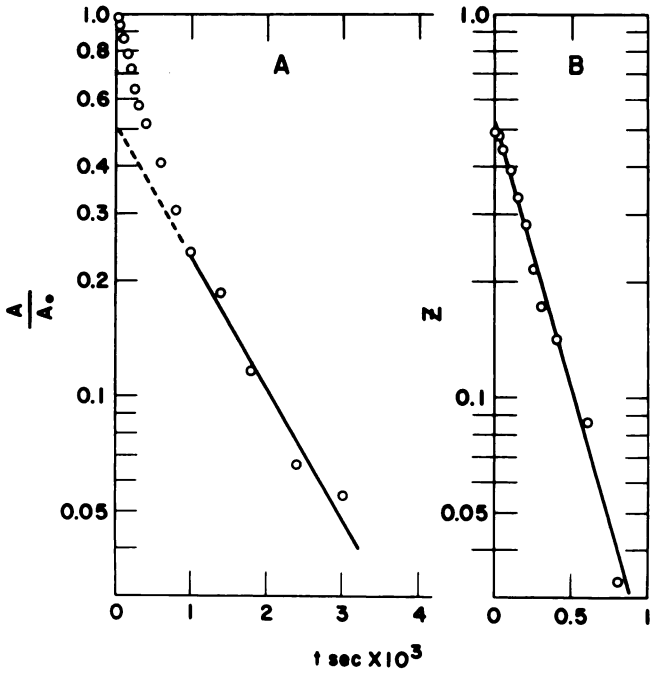


FIG. 4. *Inhibition by carb of the two types of toxin binding sites created after membrane treatment with butanol*

Entire time course of toxin binding. Membranes were treated for 30 min in the presence of 0.1 M butanol and 0.5 μM carb prior to addition to [^{125}I]α-Bgt and assayed for the entire time course of toxin binding. Concentrations of toxin binding sites and [^{125}I]α-Bgt were identical with those of Fig. 2. The *line* drawn is a least-squares fit of the time points obtained at times 1000 sec or more after initiation of the toxin binding reaction and gives $k_s = 7.83 \times 10^{-4}$ sec^{-1}.

B. Replot of the rapid phase of toxin binding. The slow phase of toxin binding is subtracted from the data at short times after initiation of the reaction and the resultant data are plotted versus t. Least-squares analysis of this line gives $k_f = 3.31 \times 10^{-3}$ sec^{-1}.

DISCUSSION

Volatile anesthetics and aliphatic alcohols affect the conformation of the membrane-bound AcChR in two characteristic ways. Their ability to stabilize preferentially the AcChR in the high-affinity state for agonists by serving as an allosteric effector was described in the accompanying communication (1). In this communication, an additional feature of the conformational perturbation of the AcChR by volatile anesthetics has been reported. In addition to stabilizing the high-affinity conformer of the AcChR, the anesthetics cause an asymmetry in this protein conformer characterized by a biphasic rate of binding of [^{125}I]α-Bgt to the protein both

in the presence and absence of agonist. In contrast, the tightly binding form of the protein produced in aged membrane preparations or generated by incubation with carb and allowed to react with toxin in the presence of the agonist can be fully described by a single exponential (1).

With the exception of halothane and octanol, the concentrations of perturbants which induced the biphasic toxin binding curves correspond to those concentrations which induce the affinity conversion. Halothane is significantly more effective in causing the affinity conversion than in inducing the biphasic curves of toxin binding. Its efficiency in promoting the affinity conversion is emphasized by its deviation in a linear free-energy relationship constructed by plotting the logarithm of the concentration required to stabilize the high-affinity form of the AcChR versus the membrane/partition coefficient (1). These data indicate that the binding locus for anesthetics possesses sufficient structural specificity such that its properties cannot be fully characterized by indices of hydrophobicity.

The alteration of the toxin binding kinetics by anesthetics provides useful insight into the structural properties of the AcChR. Strictly speaking, the biphasic toxin-binding kinetics can be interpreted in terms of the anesthetic differentially affecting two binding sites which are intrinsically asymmetrical or inducing a negatively cooperative coupling between the two sites. However, because of the emerging body of evidence favoring preexisting asymmetry, the former explanation appears most plausible. The most compelling experiment favoring the asymmetry of toxin binding sites comes from comparing the stoichiometry of toxin binding to that of the affinity labeling reagent MBTA, which reacts with a unique sulfhydryl group on the AcChR after reduction with dithiolthreitol (3, 4). There is only one site of reaction for the affinity-labeling reagent for every two toxin binding sites in the 250,000 mol wt AcChR, even though toxin protects against MBTA labeling. Since the AcChR labeled with MBTA retains the ability to bind one mole of toxin (4), the stoichiometry of the AcChR for these two specific, tightly binding ligands can be most simply explained in terms of the toxin binding sites being dissimilar and only one of them containing the locus of attachment for MBTA. Derivatization of more than one of the subunits of the AcChR by photolabile α-bungarotoxin derivatives (14) and the carb-induced efflux of sodium in microsacs prepared with AcChR labeled with MBTA are consistent with the intrinsic asymmetry of ligand binding sites (15).

Further evidence for this conclusion is provided by the demonstration that *d*-tubocurarine, a potent cholinergic antagonist, binds to different sites on the AcChR with distinct affinities (K_d = 20 nM and 7 μM) (16). Each site is present in the ratio of one per two α-neurotoxin sites, is blocked from binding *d*-tubocurarine by pretreatment with α-Bgt, and interacts with carb and the fluorescent ligand 1-(5-dimethylaminonaphthalene-1-sulfonamido)-ethane-2-trimethylammonium (16). It is interesting to note in passing that acetylcholinesterase similarly has two distinguishable binding sites for *d*-tubocurarine whose properties are sensitive to microscopic environment (17). Possibly these shared properties are evidence of the common evolutionary origins of these proteins.

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