Relationship between Reversible Antagonist Occupancy and the Functional Capacity of the Acetylcholine Receptor*

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This study examines the mechanism of action of reversible antagonists of the acetylcholine receptor, focusing on the relationship between antagonist binding and inhibition of the agonist-induced permeability response. Using intact BC3H-1 clonal muscle cells, measurements of competition between antagonists and [3H]-labeled α-toxin for surface receptors reveal Hill coefficients between 0.5 and 0.9 for antagonist association, showing that antagonists combine reversibly with multiple receptor sites of different affinity. Competition data are fit by a minimal model in which antagonists combine with two sites of equal number, A and B, that have affinities which can be distinguished prior to ligand association. The computed intrinsic dissociation constants, $K_A$ and $K_B$, yield selectivity ratios, $K_B/K_A$, ranging between 4 for alcuronium and 89 for dimethyl-$d$-tubocurarine. Parallel measurements of inhibition of the initial rate of carbamylcholine-induced $^{22}Na^+$ influx show Hill coefficients for functional antagonism between 0.9 and 1.2. When $K_B/K_A$ approaches unity, the antagonist can block the response by combining with either the A or B site. In contrast, when $K_B/K_A$ is large, as with dimethyl-$d$-tubocurarine, the antagonist blocks the response by combining primarily with the high affinity A site. The results show the two distinct sites are confined primarily to one functional receptor oligomer. In the absence of competing ligands, α-toxin shows no preference for binding to the A or B site, but in the presence of the highly selective antagonist, dimethyl-$d$-tubocurarine, toxin combines preferentially with the low affinity, B site. After α-toxin combines selectively with half of the available receptor sites, residual sites not occupied by toxin have an affinity for the reversible antagonist coincident with the high affinity dissociation constant of the original two-component binding function. When bound selectively, α-toxin blocks the response more effectively than when it combines with an equal number of sites in the absence of the selective ligand. Thus, functional surface receptors contain at least two initially distinguishable binding sites, both of which must be available to the agonist to elicit a permeability response.

In vertebrate skeletal muscle, acetylcholine combines with the postsynaptic receptor leading to an increase in the permeability of the membrane to small cations (1, 2). Classical antagonists such as $d$-tubocurarine or gallamine rapidly block the postsynaptic permeability increase through competitive inhibition of acetylcholine binding (3–5).

Consistent with a simple competitive mechanism, $d$-tubocurarine decreases the frequency of agonist-mediated channel opening events without altering the rate of closing or the single channel conductance (6, 7).

Agonists, antagonists, and elapid α-toxins bind to the same receptor subunit (8–10) and their binding is mutually exclusive (11). Antagonist binding, however, cannot be described as the reversible association with a single class of sites. Invariably, analysis of antagonist binding reveals Hill coefficients which are substantially less than unity (11–14). Several simple mechanisms might account for these low Hill coefficients: (i) each receptor oligomer contains multiple distinct sites which differ in antagonist affinity; (ii) multiple populations of receptor oligomer exist whose subunits are intrinsically equivalent, but each population differs in antagonist affinity; (iii) each receptor contains multiple sites that are initially indistinguishable but diverge in binding affinity when the sites are progressively occupied by antagonist. Any of these mechanisms could give rise to competitive functional antagonism. Each mechanism, however, predicts a distinct relationship between the occupation of receptor sites by antagonist and inhibition of the permeability response. The present experiments center upon analysis of this relationship.

Using the intact clonal muscle cell, BC3H-1, we recently examined the functional capacity of receptors in which the sites were irreversibly occupied by cobra α-toxin (13). The relationship observed between the fraction of sites occupied by α-toxin and the capacity for receptor activation was closely described by a model in which the minimal functional receptor unit contains two sites for α-toxin association and activation is blocked when α-toxin occupies either site. In addition, the two sites appear intrinsically different in their binding affinities for reversible agonists and antagonists, and this intrinsic difference preexists ligand binding. In the present work, a series of reversible antagonists are examined which span a wide spectrum in their degree of selectivity for the two sites. We measure simultaneously receptor occupancy and inhibition of the permeability response for each antagonist. The results extend our previous studies of the inhibitory capacity of bound toxin molecules (13), and examine whether the two different sites exist primarily on one functional receptor unit, or on two discrete populations of functional receptor. The data also distinguish whether receptor activation is blocked when the antagonist occupies the high or the low affinity site, either site, or both sites.

**EXPERIMENTAL PROCEDURES**

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lowing sources: Dimethyl-d-tubocurarine iodide (metacurine iodide) from Eli Lilly, Indianapolis; pancuronium bromide from Organon Inc., NJ; alcuronium (didynortoxiferine dichloride) from Hoffman-La Roche, Basel, Switzerland; AHS165 (1',1'-azoib-3-methyl-2-phenyl-1H-imidazo [1,2-a] pyridinium) from Dr. R. T. Brittain, Allen and Hanburs, Ltd., England (30). Pure cobra a-toxin was isolated from Naja naja siamensis venom from which cobramonoiodo a-toxin was purified and separated from noniodinated and diiodo-species by isoelectric focusing as previously described (15). Radiouenes, [125] and [22Na] were purchased from New England Nuclear.

Cell Cultures—Stock and experimental cultures of BC3H-1 cells were maintained as described previously (13, 14). In brief, experimental cells were seeded from stock cultures into 35-mm tissue culture dishes, and maintained in a growth medium containing a 3:1 mixture of DMF and F-12 supplemented with 8% fetal calf serum and 2% horse serum. The growth medium was replaced by fresh medium (1.5 ml) on day 14, and 1.5 ml was added again on day 16. The resulting differentiated cells were used in experiments between days 17 and 19.

Kinetics of a-Toxin Binding and Competition with Cholinergic Ligands—Experiments were performed at 21°C or 35°C in a temperature-controlled room. The depolarizing assay buffer contained: 140 mM KCl, 5.4 mM NaCl, 1.8 mM CaCl2, 1.7 mM MgSO4, 1 mM Na2HPO4, 5.5 mM glucose, 25 mM (2-hydroxymethyl)-1-piperazineethanesulfonic acid, 0.06 mg/ml of bovine serum albumin, with the addition of 11 mM NaOH to establish a pH of 7.4. a-Toxin binding and [22Na] flux measurements were performed as described previously (13, 14), using this depolarizing buffer. Briefly, cells bearing surface receptors were successively rinsed free of growth media (one 6-ml rinse), treated with 1 ml of assay buffer, and incubated at 21°C (10 min) or cooled slowly from 21 to 3.5°C (30 min). A specified concentration of cholinergic ligand was then added 20 min prior to starting the competition assay in which the incubation solution is replaced by an identical solution supplemented with [125]labeled a-toxin (10 to 20 nM). After a prescribed interval (60 s for experiments at 21°C and 120 s for 35°C), free radioligand was determined by successive washes (three 6-ml rinses at 21°C or five 6-ml rinses at 35°C). The rate constant for a-toxin binding, k1, was determined in terms of a bimolecular association between toxin and receptor using the measured specific binding and free toxin and receptor concentrations (14). The dependence of k1 on competing ligand concentration was analyzed empirically by the method of Hill (16), yielding a Hill coefficient, n, and the concentration of free ligand that reduces k1 by 50%, K0.1 (14).

Experimental points between 10% and 90% saturation were plotted according to the linear Hill equation, and regression analysis provided the values, K0.1 and n.

Carbachol-mediated Permeability Increase to [22Na] and Inhibition by Cholinergic Antagonists—The initial rate of carbachol-stimulated [22Na] influx was monitored at 35°C under conditions identical with those used for a-toxin binding (13, 14). Cells were incubated in the presence or absence of specified concentrations of cholinergic antagonists for 20 min prior to monitoring the initial rate of [22Na] influx which was elicited by 30 μM carbachol (influx was measured during a 75-s interval). The permeability to sodium ions, k0, was determined from the measured specific uptake and the equilibrium uptake which reflects the capacity of the freely exchangeable intracellular volume (13, 14). Specific uptake was analyzed in terms of a first order exchange process as described previously (13, 14). In the absence of antagonist, the rate constant, termed k0, reflects the permeability increase, and its dependence on antagonist concentration was analyzed as described above for the inhibition of the initial rate of a-toxin binding by antagonists. Similarly, linear Hill analysis yields the Hill coefficient, n, and the ligand concentration that reduces k0, by 50%, K0.5.

RESULTS

Two-site Model for Antagonist Binding—A two-site model can describe the reversible association of cholinergic antagonists which typically exhibit Hill coefficients less than unity. Previous experiments provide strong evidence that nonequivalent sites for antagonist binding can be distinguished before the initial binding event, and nonequivalence does not arise from negative cooperativity in binding (13). Hence, the negative cooperativity mechanism will not be developed quantitatively, but it will be discussed when it is clearly tested by the experiments. As monitored by ligand competition with the initial rate of [125]labeled a-toxin binding, the reversible association of an antagonist with equal numbers of two different receptor sites predicts that the initial rate of a-toxin association is described by:

\[ k_0\alpha/k_0 = 0.5(1 - x_a) + 0.5(1 - x_d) \]  

\[ k_0\alpha/k_0 = 0.5 \frac{K_a}{K_a + L} + 0.5 \frac{K_d}{K_d + L} \]  

where \( x_a \) and \( x_d \) represent the fractional occupancy of the two sites (A and B) by the antagonist, L, and equal, respectively, \( L/ (K_a + L) \) and \( L/(K_d + L) \). \( K_a \) and \( K_d \) are the intrinsic dissociation constants of the reversible antagonist for the two sites; and \( k_{aw} \) and \( k_d \) are the initial rates of a-toxin binding in the presence and absence of antagonist, respectively. As described by Equations 1 and 2, the antagonist associates with two different sites which are present in equal number, whether the different sites co-exist in one receptor oligomer or distribute, forming multiple populations of receptors. Previous experiments established that a-toxin association rates do not distinguish the two different sites for antagonist binding. a-Toxin simply binds at a rate proportional to the total number of available sites (13, 14).

Two-site Models for Functional Antagonism—Ideally, a description of functional antagonism would start with an expression for receptor activation and then include potential molecular mechanisms of antagonist action (17). At present, the precise mechanism underlying receptor activation is not well understood. However, tracer ion flux (13, 18) and electrophysiological measurements (19, 20) have established that activation requires the association of 2 or more agonist molecules. In addition, the elementary functional receptor unit apparently contains two toxin binding sites, and detectable activation results when both sites are free to combine with the agonist (13). Therefore, a simple model emerges in which each receptor contains two different sites and binding to each site is mutually exclusive for agonist, antagonist, and a-toxin. Activation would then result only when both sites are simultaneously available to the agonist (Model 1, Fig. 1). It is noteworthy that a wide variety of molecular schemes for receptor activation would simplify to this general description (17). If the two sites are initially distinguished by the agonist (as described by Equation 2), the fractional permeability response, k0/k0, is given in terms of Model 1:

\[ k_0/k_0 = (1 - x_a)(1 - x_d) \]  

Fig. 1. Two-site models depicting the functional receptor and the relationship between antagonist occupancy and inhibition with the permeability response. In Model 1, each receptor contains two ligand binding sites each of which have a different antagonist affinity. Both sites must be simultaneously available to the agonist if the receptor is to be functional. In Model 2, two populations of receptor exist, each of which is internally homogeneous but has a different antagonist affinity. Both homologous sites of either receptor subtype must be simultaneously available to the agonist for activation to occur.
Antagonist Occupancy and Functional Antagonism

The presence of the antagonist: related differently for each model. Fig. 2 shows the relationship between the concentration dependence of antagonist binding and functional antagonism for each model. In Fig. 2A, the affinities of the two binding sites are nearly equal, while in Fig. 2B they differ by 50-fold. Models 1 and 2 both predict that the degree of functional antagonism is substantially different than the corresponding saturation of sites. When the affinities of the two sites are similar, both models predict nearly equivalent degrees of functional inhibition. Moreover, with identical sites, functional antagonism exhibits slight but detectable apparent positive cooperativity. If the two sites differ greatly in affinity, as in Fig. 2B, the two models are distinguished readily. For Model 1, antagonism would follow a simple mass action relationship because function is blocked largely by ligand binding at the high affinity site. For Model 2, the antagonist blocks the response of each receptor population independently, but the overall inhibition profile is the sum of the contributions of both functional receptor populations.

Relationship between Antagonist Occupancy and Functional Capacity of the Receptor—To determine the functional capacity of the receptor in the presence of a reversible antagonist, several technical criteria should be met. As emphasized in previous studies (13, 14), the initial rate of agonist-induced sodium uptake must be measured substantially before the external tracer equilibrates with the intracellular volume. With carbamylcholine at 30 μM, 22Na⁺ tracer exchanges with about 20% of the rapidly exchangeable internal cell volume in the standard 75-s assay interval. Furthermore, since desensitization decreases the number of activatable receptors and develops more slowly in the presence of the antagonist (21), the extent of desensitization should be negligible over the duration of the assay. Therefore, permeability measurements were always performed at 3.5 °C, a temperature which maintains more than 95% of the receptors in their original activatable state for up to 2 min in the presence of 30 μM carbamylcholine (13). Since agonists and antagonists compete reversibly for a common receptor site, a minimal concentration of carbamylcholine (30 μM) was selected because it would not displace a significant fraction of bound antagonist, yet would elicit a readily detectable test response. At 30 μM, carbamylcholine occupies 5 to 10% of the receptors in the low affinity state and elicits a response of 5 to 15% of the measured maximum (cf. 13). When developing the assay to measure functional antagonism, concentrations of agonist between 18 μM and 100 μM were tested to elicit the reference response (cf. 13). The resulting apparent dissociation constants for functional antagonism increased by only 10% for the 100 μM as compared with the 18 μM reference. Thus, at 30 μM, carbamylcholine does not significantly displace the reversible antagonist, violate the initial rate condition for tracer influx, or elicit desensitization under the conditions used in the following experiments.

A series of reversible antagonists were examined for their concentration dependence of competition with the initial rate of 125I-labeled α-toxin binding and the inhibition of the initial rate of carbamylcholine-stimulated 22Na⁺ uptake (Fig. 3). Superimposed upon the experimental competition data are best fit curves for the reversible association of the antagonist with equal numbers of two distinct binding sites (Equation 2). The measured binding functions were initially analyzed in terms of the empirical Hill equation (cf. 14) giving apparent dissociation constants, Kᵦ, and Hill coefficients, n (Table I). For all antagonists, values of n were less than unity, warrant-

\[
k_\text{a}/k_\text{sa} = \frac{K_\text{a}}{K_\text{a} + L} \frac{K_\text{b}}{K_\text{b} + L} \\
k_\text{d}/k_\text{sd} = 0.5 \left( \frac{K_\text{a}}{K_\text{a} + L} \right)^2 + 0.5 \left( \frac{K_\text{b}}{K_\text{b} + L} \right)^2
\]
Antagonist Occupancy and Functional Antagonism

Fig. 3. Concentration dependence for antagonist inhibition of the initial rate of \(^{38}\)I-labeled α-toxin binding and inhibition of the carbamylcholine-mediated permeability increase to \(^{36}\)Na\(^{+}\). Cells were covered with buffer, cooled slowly (30 min) to 3.5°C, and incubated for 20 min in the presence of the specified concentrations of antagonist. Antagonist occupation was then measured by its competition with the initial rate of α-toxin binding which was determined in a 120-s interval (squares) and is expressed relative to the control rate, \(k_r\), determined in the absence of antagonist. Functional antagonism was measured by replacing the prior incubation solution with an identical solution supplemented with 30 μM carbamylcholine and \(^{36}\)Na\(^{+}\), after which the initial rate of tracer sodium uptake was monitored in a 75-s interval (circles). The resulting permeability change, \(k_{\text{diss}}\), is calculated in terms of a first-order exchange of isotope (cf. Ref. 14) and is expressed relative to \(k_{\text{diss}}\) measured in the absence of antagonist. Each experimental point is the mean of duplicate determinations. The solid curve associated with the squares is the best fit of Equation 2 to measurements of antagonist binding, and best fit values of \(K_A\) and \(K_B\) are listed in Table I. The remaining solid and dotted curves are, respectively, the predictions for functional antagonism in terms of Model 1 or Model 2, using the values \(K_A\) and \(K_B\) which are derived independently from analysis of antagonist binding (Table I).

Table I. Parameters for antagonist competition with α-toxin binding and inhibition of the permeability increase elicited by carbamylcholine

| Antagonist          | \(K_A^a\)  | \(n^a\)  | \(K_A^b\)  | \(K_D^b\)  | \(K_D/K_A\) | \(K_B/K_B^a\) | \(n^b\)  | \(K_{\text{Diss}}/K_{\text{Diss}}^a\) |
|---------------------|-----------|---------|------------|------------|-------------|-------------|---------|-------------------------------|
| Alcuronium          | 4.2 × 10\(^{-5}\) | 0.87 ± 0.03 | 2.14 × 10\(^{-4}\) | 8.68 × 10\(^{-5}\) | 4.1 | 1.3 × 10\(^{-2}\) | 0.99 ± 0.02 | 3.3 |
| Pancuronium         | 2.3 × 10\(^{-4}\) | 0.86 ± 0.02 | 9.11 × 10\(^{-4}\) | 6.93 × 10\(^{-5}\) | 7.6 | 7.4 × 10\(^{-5}\) | 1.16 ± 0.07 | 3.2 |
| AHS165⁵             | 6.0 × 10\(^{-5}\) | 0.78 ± 0.01 | 1.53 × 10\(^{-4}\) | 1.84 × 10\(^{-5}\) | 10.3 | 2.1 × 10\(^{-5}\) | 1.08 ± 0.03 | 2.9 |
| Gallamine           | 1.5 × 10\(^{-5}\) | 0.70 ± 0.03 | 3.70 × 10\(^{-5}\) | 5.50 × 10\(^{-5}\) | 14.8 | 3.7 × 10\(^{-5}\) | 1.00 ± 0.06 | 4.0 |
| dimethyl-d-tubocurarine | 3.1 × 10\(^{-6}\) | 0.51 ± 0.03 | 3.09 × 10\(^{-7}\) | 2.75 × 10\(^{-3}\) | 89.0 | 4.7 × 10\(^{-7}\) | 0.85 ± 0.05 | 6.9 |

\(^a\) \(K_A\) is the concentration of antagonist which decreases the initial rate of toxin binding, \(k_r\) by 50% and \(n\) is the associated Hill coefficient. \(K_A\) and \(n\) are calculated by fitting the Hill equation to the data. Hill coefficients are the slopes of the best fit line (cf. Ref. 22).

\(^b\) \(K_B\) and \(K_{\text{Diss}}\) are the best fit high and low affinity intrinsic dissociation constants resulting from the fit of Equation 2 to the experimental data.

\(^c\) \(K_{\text{Diss}}\) is the concentration of antagonist which diminishes the permeability increase elicited by 30 μM carbamylcholine by 50% and \(n\) is the associated Hill coefficient.

\(^d\) See Ref. 32.
the ratio, $k_D/k_{on}$, becomes larger (Table I). Similarly, as $K_D/K_A$ increases, $K_{on}$ approaches the value for antagonist binding to the high affinity site, $K_A$. With highly selective antagonists, the Hill coefficient for inhibition of the response should approach a minimal value of $1.0$, as is observed for gallamine and dimethyl-$d$-tubocurarine. By contrast, when $K_A$ and $K_B$ are nearly equal, $K_{on}$ diverges from $K_A$ and the concentration dependence of functional antagonism should exhibit a Hill coefficient slightly greater than unity, as is most evident from the data on pancuronium.

**Influence of Selective a-Toxin Occupancy on the Binding of Reversible Antagonists**—Additional evidence is now presented supporting the two-site model which accounts for the low Hill coefficients characteristic of antagonist binding. As shown in Fig. 3, dimethyl-$d$-tubocurarine exhibits a high degree of selectivity for the two antagonist sites. In the presence of a concentration of dimethyl-$d$-tubocurarine which preferentially occupies the high affinity site, a-toxin can be directed to bind selectively and irreversibly to the low affinity site. Under these selective conditions, if more than half of the total sites were first occupied by a-toxin, nearly all of the residual sites should be those that bind the antagonist with a high affinity. To achieve selective a-toxin occupancy, a solution containing a-toxin plus $21 \mu M$ dimethyl-$d$-tubocurarine was applied to cells and a-toxin was allowed to occupy half of the total sites. Cells were washed thoroughly removing the reversible antagonist, and the binding of dimethyl-$d$-tubocurarine was monitored to the remaining sites by competition with $^{125}$I-labeled a-toxin binding. Indeed, Fig. 4 reveals the dissociation constant for dimethyl-$d$-tubocurarine binding to residual sites is shifted 10-fold from the protection constant for the entire ensemble of sites. In addition, the measured Hill coefficient approaches unity. Moreover, a two-site fit to the control binding function for dimethyl-$d$-tubocurarine (measured without prior selective toxin occupancy) yields a high affinity dissociation constant, $K_A$ which approaches the apparent dissociation constant measured for the sites remaining following selective toxin occupation. Since dimethyl-$d$-tubocurarine does not select absolutely between the two sites, a small fraction of sites not occupied by toxin would be expected to have a low instead of a high affinity. A small fraction of persisting low affinity sites could give rise to the small deviation from a unitary Hill coefficient.

When toxin binds to low affinity sites in the presence of dimethyl-$d$-tubocurarine, the competition between a-toxin and alcuronium reveals that alcuronium, like dimethyl-$d$-tubocurarine, binds to the residual sites with high affinity. Again, the apparent dissociation constant for the residual sites approaches to the $K_A$ value generated from a two-site fit to the overall binding function for alcuronium. These two antagonists apparently share the same high affinity binding site. Thus, measurements of the selective binding of antagonist to the high affinity site provide independent evidence that a two-site model accurately describes the present determinations of antagonist binding.

Either a two-site or a negative cooperativity description of antagonist binding would be consistent with the higher antagonist binding affinity which residual sites exhibit following selective occupation of receptor sites by a-toxin. The negative cooperativity mechanism, however, fails to account for previous experimental findings (13). When fractional toxin occupancy is achieved nonselectively (i.e. in the absence of dimethyl-$d$-tubocurarine), antagonist binding to residual sites is not altered (13). If the two sites were initially indistinguishable, as required by the negative cooperativity mechanism (23), both the selective and random binding of a-toxin should decrease the fraction of paired vacant binding sites which are able to exhibit putative negative cooperativity. Hence, the antagonist must associate with intrinsically different sites which are distinguishable prior to ligand binding.

**Functional Capacity of Receptors Following Progressive Degrees of Selective Toxin Occupation**—In experiments described previously (13), we presented compelling evidence that the permeability response is blocked when a-toxin combines with either site on the functional receptor unit. Hence, the relationship between the fractional receptor response, $k_o/k_{ion}$ and the fractional saturation of sites by toxin, $y$, is given by:

$$k_o/k_{ion} = (1 - y)^2$$

Since toxin distributes randomly among the nonequivalent antagonist sites, Model 1 ($\Phi \Phi$, different sizes on the same receptor) is not distinguished from Model 2 ($\bigcirc \bigcirc$ and $\bigcirc \bigcirc$, two different populations of receptors) by this relationship. How-
however, if α-toxin were directed selectively to the low affinity site, each model predicts a different relationship between the fractional response and the fractional saturation of α-toxin sites. In particular, if the different sites exist on two populations of receptors, in the presence of a selective antagonist, hybrid species which carry 1 toxin molecule would be generated randomly within each receptor population, and for Model 2 the fractional response, \( k_c/k_c, \) as a function of the fraction of sites occupied by α-toxin, \( y, \) is described by:

\[
k_c/k_c = 0.5(1 - y)^2 + 0.5(1 - y) < (1 - y)^2
\]  

Equation 8. \( y_A \) and \( y_B \) represent the fraction of each distinct binding site which is occupied by α-toxin. If the two different sites were on one receptor, however, formation of hybrid species (as opposed to species doubly occupied by α-toxin) would be strongly favored, causing a greater reduction in response for a given degree of α-toxin occupation. If toxin occupies sites in the presence of a selective reversible antagonist, for Model 1 the fractional response as a function of fractional toxin occupancy is described by:

\[
k_c/k_c = (1 - y_A)(1 - y_B) < (1 - y)^2
\]  

Equation 9. Damle and Karlin (24) have used an extension of Equation 9 to describe the hypothetical case in which α-toxin associates with two sites on the receptor at two intrinsically different rates.

Progressive numbers of receptor sites were occupied by \(^{125}\text{I}-\alpha\)-toxin by incubating cells for increasing durations with α-toxin, in the presence (selective) or in the absence (nonselective) of dimethyl-d-tubocurarine. Cells were thoroughly washed to remove dimethyl-d-tubocurarine, and the receptors were examined for functional capacity by monitoring the initial rate of carbamylcholine-mediated sodium uptake. As shown previously, when α-toxin associates randomly with receptor sites the relationship between fractional response and toxin occupation is closely described by Equation 7 (Fig. 5). In contrast, when toxin binds in the presence of the selective antagonist, its binding inhibits the permeability response more effectively than when it combines with sites in the absence of the antagonist. Since Model 1 predicts that the degree of functional antagonism would be greater for the selective as compared with the random toxin labeling condition (see Equation 9), this experiment provides further support that the two different binding sites exist largely on one functional receptor. Quantitatively, the reduction in response achieved under selective conditions is somewhat less than would be anticipated based on the 13- to 19-fold selectivity for α-toxin binding to the low affinity site which 21 μm dimethyl-d-tubocurarine should confer (cf. 24). Potential sources of uncertainty include:

(i) The selectivity calculated is the ratio of low to high affinity sites available to toxin in the presence of dimethyl-d-tubocurarine, and could be overestimated because the calculation is based on fitted values of \( K_A \) and \( K_B \) generated from a separate experiment (Fig. 3, top). (ii) Hybrid species, or receptors carrying 1 toxin molecule, may elicit a low but significant permeability increase. If hybrid species would elicit a low but detectable response, point ii may also account for the small deviation from Equation 7 which is evident when toxin binds randomly to receptor sites (Fig. 5 and Ref. 13).

**DISCUSSION**

Occupation of the cholinergic receptor of intact BC3H-1 cells by several reversible antagonists results in a nonrandom distribution of occupied sites among the surface receptors. Antagonists bind nonrandomly because two sites in the receptor population possess intrinsically different affinities which are distinguishable prior to antagonist binding. The two models shown in Fig. 1 represent the simplest limiting cases which potentially account for the relationship between antagonist binding and functional antagonism. In Model 1, the nonequivalent sites are confined to one functional receptor oligomer (\( \text{C} \)), and in Model 2, equivalent binding sites pair forming two distinct populations of functional receptor oligomers (\( \text{C} \) and \( \text{C} \)). The data clearly show a preferred fit to Model 1. Hence, within experimental uncertainty, the majority of functional receptors apparently contains two nonequivalent sites. Nevertheless, the present data alone cannot eliminate the possibility that a small population of receptors contains equivalent binding sites. For example, three dimeric species (\( \text{C} \), \( \text{C} \), \( \text{C} \)) may form by the pairing of equivalent or nonequivalent sites; however, their frequency of occurrence could not be predicted \( \text{a priori} \). A model involving three species would introduce additional free parameters, and would be expected to provide a closer fit to the measurements of functional antagonism, particularly those for dimethyl-d-tubocurarine (see Fig. 3).

The observed relationship between occupancy of receptor sites by reversible antagonists and functional antagonism supports and extends previous studies of the inhibitory capacity.
of irreversibly bound α-toxin molecules (13).\(^1\) α-Toxin and reversible antagonists abolish the response through association with one of two sites on the functional receptor unit. The essential difference between α-toxin and reversible antagonists is that α-toxin labels the available sites at random, while for some reversible antagonists occupation of one of the two sites is heavily favored because of the intrinsic difference in dissociation constants. For the ligands pancuronium or alcuronium, which have nearly equal affinities for the two sites, functional inhibition results when either site becomes occupied by antagonist. In contrast, because of its high degree of selectivity, dimethyl-d-tubocurarine blocks receptor activation largely by combination with the high affinity site. Thus, reversible antagonists, like α-toxin (13), reveal that at least two sites control the function of each receptor since both sites must be available to the agonist in order to elicite a permeability increase.

It is now widely recognized that antagonist occupation of the receptor involves the association with at least two sites possessing different affinities. Hill coefficients range from 0.5 to 0.9 for the binding of rigid planar antagonists as measured by competition with the initial rate of α-toxin binding to receptors of Torpedo membranes (12) or intact BC3H-1 cells (13, 14). In addition, Neubig and Cohen (11) monitored binding of the reporting ligands, danylabeled acetylcholine, and examination of these ligands by the antagonists, d-tubocurarine, dimethyl-d-tubocurarine, and gallamine. Their measurements also detected antagonist binding at two discrete sites in Torpedo membranes. Moreover, direct measurements of d-tubocurarine binding revealed equal numbers of two sites which bind the antagonist with a 500-fold difference in affinity. For the mammalian receptor from BC3H-1 cells, it has recently become clear that when receptor species containing a single bound toxin molecule are formed by the random associated of α-toxin, agonists also reveal intrinsic differences in the binding affinity of each site (13). Curiously, α-toxin is the only ligand that shows no detectable preference for binding to either receptor site. Inspection of Table I reveals that reversible antagonists with an extremely high affinity show only a small degree of selectivity for binding at the two sites. Thus, α-toxin represents the extreme ligand of this trend, showing no measurable selectivity for the two sites.

In a classical study of functional antagonism by d-tubocurarine, Jenkinson (1) observed that d-tubocurarine causes a parallel shift of the dose-response relationship for receptor activation toward higher agonist concentrations. Subsequently, this general feature of competitive antagonism by d-tubocurarine has been confirmed (5, 26). In further support of an action at the agonist recognition site, fluctuation (7) and voltage jump relaxation analyses (6) indicate that d-tubocurarine reduces the frequency of channel opening events without altering the closing rate or the single channel conductance.

However, recent voltage clamp experiments have uncovered an apparent noncompetitive action of d-tubocurarine at frog endplate receptors (26). At negative membrane potentials, noncompetitive antagonism is seen along with a predominant competitive component. The authors suggest that noncompetitive antagonism results from the voltage-dependent entry of d-tubocurarine into the mouth of the ion channel, in turn blocking the passage of sodium ions. In Aplysia neurons (27), d-tubocurarine apparently blocks the acetylcholine response solely through a voltage-dependent channel-blocking mechanism. In the present experiments, the membrane potential is maintained near 0 mV by using a high concentration of extracellular potassium. Thus, if a voltage-dependent channel block is operative in the receptors on BC3H-1 cells, it should not be favored by the depolarizing conditions used in these experiments. Moreover, since the relationship between occupation and functional antagonism is described well by Model 1, additional sites of action are not evident. Still, in BC3H-1 cells, independent evidence is required to disprove unequivocally an antagonist action at a second site.

Drug antagonism is interpreted classically using the Schild analysis (28), often termed "the null method." This analysis is commonly employed to test for a competitive mechanism for inhibition and to determine the antagonist dissociation constant. The ratio of antagonist concentrations required to obtain equivalent responses in the presence and absence of antagonist is equal to \(1 + L/K_A\) where \(L\) is the antagonist concentration and \(K_A\) is the antagonist dissociation constant. The original derivation of this expression assumes that the response is noncooperative and channel opening results when one agonist and one receptor combine reversibly forming the active, agonist-receptor complex. Colquhoun (17) considered the predicted Schild plot when cooperative activation occurs and has shown that for some plausible schemes, the reversible binding of antagonist at the agonist recognition site still results in the anticipated properties of competitive antagonism. Indeed, in several systems, d-tubocurarine causes a parallel shift of the dose-response curve for receptor activation, and Schild analysis yields a single dissociation constant (3, 5, 26). In light of the present experiments, the dissociation constant determined from Schild analysis would differ substantially from the apparent dissociation constant for antagonist binding.

Thus, for agents with a high degree of selectivity for the two sites, the dissociation constant generated from functional antagonism \((K_{ant})\) equals the intrinsic dissociation constant for binding to the high affinity site. In contrast, nonselective agents (i.e. \(K_B/K_A = 1\)) would exhibit an apparent dissociation constant for functional antagonism, \(K_{ant}\), which is smaller than the intrinsic dissociation constants for antagonist binding (in Model 1, when \(K_{app} = K_A = K_B; K_{ant} = K_{app}(1 + \sqrt{2}) = K_{app}(2.414)\)).

The present demonstration that the functional receptor contains at least two distinguishable sites for antagonist association provides information complementary to recent structural studies. The minimal structural receptor unit from Torpedo is a 250,000-dalton pentamer which contains four polypeptide chains, \(\alpha, \beta, \gamma, \delta\), in the stoichiometric ratio \(\alpha\beta_2\gamma\delta_2\) (29, 30). Since the \(\alpha\) subunit is the primary locus for agonist (8), antagonist (9), and α-toxin association (10), this basic set of subunits most likely represents the functional receptor unit. Intrinsic nonequivalence in the \(\alpha\)-subunits is not evident since they have identical electrophoretic migration and NH₂-terminal primary sequences (31). However, only one of the two \(\alpha\) subunits will react with the sulphydryl affinity labeling reagents, MBTA and bromacetylcholine (29). Under certain conditions, bromacetylcholine will react with both subunits but at different rates (33). This difference in reactivity, however, is evident only following reduction of the receptor, and may simply reflect a more occluded sulphydryl group in one of the \(\alpha\) subunits. Reversible agonists and antagonists clearly distinguish two different binding sites, and these sites appear...
to be on the same functional receptor unit. Since the compositions of the two a-subunits appear chemically identical, the nonequivalence revealed by ligand binding could arise from dissimilar intersubunit contacts. Each a-subunit would necessarily be exposed to a different set of intersubunit contacts because of the αβγδ, stoichiometry, since the subunits are intrinsically asymmetric, and they span the bilayer. Identification of the molecular basis of the nonequivalence in the two ligand recognition subunits is an essential, unanswered question. Answers to this question should provide insight into other aspects of receptor function, including mechanisms of receptor assembly, the role of the other subunits in receptor function, and mechanisms underlying the cooperative response.

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