Monoclonal Antibodies Specific for Each of the Two Toxin-Binding Sites of *Torpedo* Acetylcholine Receptor†

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ABSTRACT: We have isolated and characterized 12 monoclonal antibodies (mAbs) that block the binding of α-bungarotoxin (α-BuTx) to the acetylcholine receptor (AChR) of *Torpedo californica*. Two of the mAbs block α-BuTx binding completely; the other 10 inhibit only about 50% of the binding. The mAbs that partially inhibit α-BuTx binding can be divided into two groups by examination of the additive effect of pairs of mAbs on toxin binding, and by analysis of competition between mAbs for binding to the AChR. These two groups of mAbs, which we have termed A and B, appear to recognize different toxin-binding sites on the same receptor. A and B mAbs were used to determine the kinetic and pharmacological properties of the two sites. The site recognized by A mAbs binds α-BuTx with a forward rate constant of 0.98×10^5 M^{-1} s^{-1}, *d*-tubocurarine (dTC) with a K_D of $(6.8 \pm 0.3) \times 10^{-8}$ M, and pancuronium with a K_D of $(1.9 \pm 1.0) \times 10^{-9}$ M. The site recognized by B mAbs binds α-BuTx with a forward rate constant of 9.3×10^5 M^{-1} s^{-1}, dTC with a K_D of $(4.6 \pm 0.3) \times 10^{-6}$ M, and pancuronium with a K_D of $(9.3 \pm 0.8) \times 10^{-6}$ M. Binding of A and B mAbs to the AChR was variably inhibited by nicotinic cholinergic agonists and antagonists, and by α-conotoxin. The observed pattern of inhibition is consistent with the relative affinity of the two sites for antagonists as given above but also indicates that the mAbs recognize a diversity of epitopes within each site.

The nicotinic acetylcholine receptor (AChR)[1] is a pentameric protein composed of four different subunits in the stoichiometric ratio $\alpha_2\beta\gamma\delta$ (Karlin, 1980; Conti-Tronconi & Raftery, 1982; Changeux et al., 1984; McCarthy et al., 1986). Each of the subunits traverses the membrane; assembled, they form a cation-specific channel at their center (Kistler et al., 1982). The ion channel is opened by binding of the neurotransmitter acetylcholine (ACh) to two sites that are associated with the two α subunits (Weill et al., 1974; Wolosin et al., 1980). Opening of the channel normally requires binding of ACh to both sites (Dionne et al., 1978; Sine & Taylor, 1980), but infrequent and brief channel openings can occur after binding of a single ACh molecule (Labarca et al., 1985).

Several observations suggest that the two ACh-binding sites of the receptor may have intrinsically different properties. Reversible antagonists, such as *d*-tubocurarine, bind to the AChR with two different affinities (Neubig & Cohen, 1979; Sine & Taylor, 1981; Gu et al., 1985; Hamilton et al., 1985), and the reduction and alkylation of a disulfide bond near the binding site for ACh occur with different facility at the two sites within each AChR (Damle & Karlin, 1978; Wolosin et al., 1980; Walker et al., 1984). Experiments with α-neurotoxins, which bind at or near the ACh-binding sites, also indicate that there may be intrinsic differences between the two sites. The binding reaction for α-neurotoxins has in some cases been reported to have two kinetic components (Brockes & Hall, 1975; Maelicke et al., 1977; Blanchard et al., 1979; Mihovilovic & Richman, 1984), and several groups have reported that monoclonal antibodies or antibodies from myasthenic sera block one of the two binding sites for α-bungarotoxin (Watters & Maelicke, 1983; Mihovilovic & Richman, 1984; Gu et al., 1985; Whiting et al., 1985).

To study the functional role of the two sites and the structural basis of their differences, we have prepared a set of 12 monoclonal antibodies (mAbs) that block the binding of α-BuTx to the *Torpedo* AChR. Six of these mAbs specifically block binding of α-BuTx to one of the sites, four block binding to the other site, and two block binding to both sites. We report here experiments characterizing these antibodies and using them to relate the immunological, pharmacological, and kinetic properties of the two toxin-binding sites of the AChR. Our studies provide strong evidence that the two sites are intrinsically different in the intact receptor.

Experimental Procedures

Materials

Torpedo californica was obtained from Pacific Biomarine (Venice, CA), and the electric organs were removed and stored at −70 °C. BALB/C and C57 mice were obtained from Simonson, Inc; C56 mice were from Jackson, Inc. All mice were females of about 6 weeks of age.

Rabbit antisera against mouse IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 were obtained from Miles Laboratories; goat IgG fractions against rabbit and mouse antibodies were from Cappel Laboratories. Cyclophosphamide was obtained from Adria Laboratories Inc.; pancuronium bromide was from Organon Inc; *d*-tubocurarine (dTC) was from Calbiochem; Triton X-100

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; dTC, *d*-tubocurarine; mAb, monoclonal antibody; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; DTT, dithiothreitol; PBS, 0.15 M NaCl and 10 mM sodium phosphate, pH 7.4; Hb, hemoglobin.

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was from Baker Chemical Co.; decamethonium, hexamethonium, carbamylcholine, α-BuTx, Tween 80 and phenol-extracted lipopolysaccharide from *Salmonella typhimurium* were from Sigma Chemical Co. Nutridoma SP was obtained from Boehringer-Mannheim. $Na^{125}I$ and ^{125}I-α-BuTx were obtained from Amersham International Corp. For experiments on the kinetics of toxin binding, ^{125}I-monoiodo-α-BuTx was prepared according to the procedures described in Wang and Schmidt (1980). α-Conotoxin G1 (Gray et al., 1981; Olivera et al., 1985) was the generous gift of Dr. Baldomero Olivera (University of Utah).

Methods

Purification of Torpedo AChR. AChR from the electric organ of *Torpedo californica* was prepared as described by Froehner et al. (1977) except that 0.1% (w/v) Tween 80 instead of deoxycholate was used during the wash and elution steps from the cobra toxin–Sepharose column; also, the eluted AChR was dialyzed against 10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, and 0.1% (w/v) Tween 80. In some experiments, the AChR was converted to the monomeric form by reduction with 10 mM dithiothreitol (DTT) for 30 min at 20 °C and alkylation with 10 mM *N*-ethylmaleimide for 30 min at 4 °C (Chang & Bock, 1977).

DEAE Filter Assay. AChR concentrations were routinely assayed by the binding of ^{125}I-α-BuTx, using the DEAE filter assay as described by Brockes and Hall (1975). Inhibition of α-BuTx binding by mAbs was measured after preincubation of the receptor with one or two mAbs at 37 °C for 90 min before addition of ^{125}I-α-BuTx. Both dialyzed hybridoma supernatants and diluted ascites fluid samples were used in these assays.

Radiolabeling of Purified AChR. Affinity-purified AChR in 10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 0.5 mM EGTA, and 0.1% Tween 80 (60 μg of receptor in 250 μL total volume) was incubated with 250 μCi of $Na^{125}I$ in the presence of 0.05 mg of Chloramine T for 1 min at 20 °C, and the reaction was quenched with 50 μL of 0.4 mg/mL tyrosine. The iodinated receptor was immediately desalted on a P6-DG column equilibrated with the same buffer and stored on ice.

Immunization Protocols. Two procedures were used to generate the desired immune response against the α-BuTx-binding sites of *Torpedo* AChR. In the first procedure, lipopolysaccharide from *Salmonella typhimurium* was reacted with cyanogen bromide and coupled to AChR by a procedure similar to that described by Lange et al. (1983); the activated lipopolysaccharide was coupled to the AChR in a solution that contained 100 mM $NaHCO_3$ (pH 8.7), 0.5 mM EDTA, 0.5 mM EGTA, and 0.01% (w/v) sodium cholate. Due to the presence of the detergent, which was required to keep the AChR in solution, it was not possible to separate free lipopolysaccharides from the AChR–lipopolysaccharide complexes. Five-microgram aliquots of this immunogen were injected into young female BALB/c mice, resulting in an excellent immune response. The spleen cells from one of these mice were given a final boost by the in vitro immunization protocol described by Pardue et al. (1983).

The second immunization protocol was based on the method described by Matthew and Patterson (1983). AChR (solubilized in 0.1% Tween 80) was incubated with a slight excess of α-BuTx to block the toxin-binding sites, then diluted in saline, emulsified with Freund's complete adjuvant, and injected intraperitoneally into 6-week-old female C57 BL mice [approximately $(1–3) \times 10^{-10}$ M α-BuTx sites per animal]. After 5 min, the mice were injected with cyclophosphamide in PBS (70 mg/kg). Further cyclophosphamide injections were given 24 and 48 h after the initial immunization. Four weeks later, the mice were immunized with AChR emulsified in Freund's complete adjuvant [$(1–3) \times 10^{-10}$ M BuTx sites per animal]. Three weeks later, intraperitoneal injections of AChR in saline containing 0.01% Tween 80 were given on three successive days (6×10^{-10} M α-BuTx sites per injection), and on the fourth day, the spleens were used for fusions.

Fusion, Screening, and Cloning of Hybridomas. Immune spleen cells were fused with SP 2/0 myeloma cells using 38% (w/v) poly(ethylene glycol) 4000, according to standard procedures. Nonimmune spleen cells were used as feeders in the 96-well culture plates, and HAT medium was used to permit the selective growth of hybridomas. Hybridoma supernatants were screened by a reverse solid-phase radioimmunoassay technique (see below). Initially, hybridomas secreting mAbs against detergent-solubilized AChR were identified; these "positive" hybridomas were then retested against AChR–α-BuTx complexes. Anti-AChR mAbs which bound AChR but not AChR–α-BuTx complexes were selected for further investigation. Cloning of positive hybrids was achieved by using the limiting dilution technique.

Purification and Iodination of mAbs. mAbs that were IgGs were purified from ascites fluids using DEAE-Affi-Gel B (Bio-Rad) affinity chromatography as described by Bruck et al. (1982) and were iodinated with Chloramine T.

Standard Reverse Solid-Phase Radioimmunoassay. Round-bottom disposable microtiter plates (Dynatech Laboratories Inc.) were used for all the solid-phase assays described in this paper, and all procedures were carried out at room temperature. For some assays, including the hybridoma screening, wells were coated with goat IgG anti-mouse immunoglobulin (IgG, IgM, and IgA) by incubation for 12 h with 30-μL aliquots of 0.08 mg/mL IgG diluted in PBS and 0.02% NaN_3. The wells were washed 3 times with 2% (w/v) bovine hemoglobin in PBS with 0.02% NaN_3, incubated for 1 h with this solution, and washed 3 more times. The wells were then incubated with 25-μL aliquots of either hybridoma supernatant or diluted ascites fluid for 4–16 h at 20 °C. The microtiter plates were washed 4 times with 2% (w/v) hemoglobin, 0.02% sodium azide, and 0.05% (w/v) Tween 80 in PBS (Hb/Tween solution), and the wells were incubated with ^{125}I-AChR diluted in Hb/Tween solution for 4–12 h before finally being washed 4 more times with Hb/Tween solution. The wells were then cut out of the plate with a hot wire and counted in a Beckman γ counter.

Binding of ^{125}I-AChR and ^{125}I-AChR–mAb Complexes to Solid-Phase mAbs. Supernatants from hybridomas cultured in serum-free medium (Nutridoma SP) were used to coat wells of microtiter plates (50 μL/well, overnight), and the wells were then blocked with Hb/Tween solution as in the standard radioimmunoassays. Iodinated AChR (8×10^{-9} M in α-BuTx sites) was incubated with excess A- or B-type mAb (1/50 dilution of ascites fluid) in Hb/Tween solution for 90 min at 20 °C and was then added to the coated wells (25 μL/well) of the microtiter plates. After 2 h of incubation, the wells were washed 4 times with Hb/Tween solution and counted as described above.

Binding of AChR–^{125}I-mAb Complexes to Solid-Phase mAbs. Wells of microtiter plates were coated with serum-free hybridoma supernatant as described above. AChR, which had been treated with 10 mM DTT and alkylated with 10 mM *N*-ethylmaleimide, was diluted in Hb/Tween solution and incubated with iodinated A- or B-type mAbs [specific activity $(2.5–7.5) \times 10^8$ cpm/mg of protein] for 1 h. Aliquots (25 μL) of the incubation mixtures were then added to the coated wells

and incubated for 150 min. Finally, the wells were washed 4 times with Hb/Tween solution and counted as described above.

Binding of α-BuTx to AChR Attached to Solid-Phase mAbs. In some experiments, wells were coated with the IgG fraction of goat anti-mouse immunoglobulin and, after blocking with hemoglobin, were incubated with mAbs. In most experiments, however, the wells were coated directly with serum-free hybridoma supernatants, as described above. The wells were then incubated with 25-μL aliquots of 3.5×10^{-9} M reduced and alkylated AChR in Hb/Tween solution for 7 h, followed by mAb ascites fluids (1/200 dilution in Hb/Tween solution) for 9 h to block vacant toxin-binding sites, and were then incubated with 5×10^{-9} M ^{125}I-BuTx (25 μL/well) for 270 min before the wells were washed 4 times and counted.

Solid-Phase Assay for Kinetic Analysis of α-BuTx Binding to AChR and AChR–mAb Complexes. The association rate for α-BuTx binding to AChR was investigated by using a solid-phase assay. Microtiter wells were coated with an mAb (14-3-F7) that binds the AChR but does not interfere with binding of α-BuTx (A. J. Dowding and Z. W. Hall, unpublished experiments). Wells were incubated with 50 μL of purified IgG (0.07–0.1 mg/mL in PBS) for 12 h at 20 °C. The wells were then blocked with Hb/Tween solution as described above and incubated with purified AChR in Hb/Tween solution (7.86×10^{-9} M in α-BuTx sites) for 1–3 h. The toxin-binding sites of the tethered AChR were subsequently blocked by incubation for 2–3 h with A- or B-type mAb (ascites fluid diluted 1/50 in Hb/Tween solution). ^{125}I-Monoiodo-α-BuTx (10^{-8} M) was added to the wells for various periods before being replaced by unlabeled α-BuTx (1.25×10^{-7} M) to quench the wells; the labeled and unlabeled toxins were diluted in Hb/Tween solution. Finally, the wells were washed 4 times with Hb/Tween solution and counted as described above. The experiments were analyzed as described by Mihovilovic and Richman (1984). The experimental data were fit to eq 2, described under Results, with nonlinear regression analysis, using a computer program adapted from that given by Yamaoka et al. (1981).

Cholinergic Ligand-Mediated Inhibition of AChR Binding to Solid-Phase mAbs. Detergent-solubilized AChR, at a final concentration of 4×10^{-9} M, was incubated with several types of cholinergic ligands for 30 min at 20 °C, in Hb/Tween solution, using a range of concentrations for each of the ligands. Twenty-five-microliter aliquots of each of the incubation mixtures were added to wells of a microtiter plate, which had been coated with mAbs, as described for the standard reverse solid-phase radioimmunoassay. After 3 h, the wells were washed 4 times with Hb/Tween solution, then incubated for 90 min with 8×10^{-9} M ^{125}I-α-BuTx, and finally washed 4 times with Hb/Tween solution, before being counted. For each mAb, AChR binding in the presence of competing ligand was compared with AChR binding in the absence of ligand. ^{125}I-AChR was also used in some experiments and gave essentially the same results as those obtained with unlabeled AChR and ^{125}I-α-BuTx.

Solid-Phase Assay for Pharmacological Analysis of the Toxin-Binding Sites of AChR and AChR–mAb Complexes. The procedure was similar to that used for the kinetic analysis of toxin binding: purified AChR was tethered to microtiter plates using mAb 14-3-F7, and the immobilized AChR was subsequently reacted with A- or B-type mAbs (1/50 dilution of ascites fluid). The wells were then incubated with the various concentrations of dTC or other cholinergic ligands at 20 °C for 30 min. Aliquots of $(2–3) \times 10^{-9}$ M ^{125}I-monoiodo-α-BuTx solution, which also contained the indicated concentration of antagonist, were then added to the wells for exactly 40 s before being replaced by quenching solution (1.25×10^{-7} M unlabeled α-BuTx in Hb/Tween solution). Finally, the wells were washed 4 times and counted. The rates of α-BuTx binding in the presence of various concentrations of antagonist were fit to an equation for a two-site model for antagonist binding as described by Sine and Taylor (1981):

$$K_{obsd}/K_t = \frac{n_A K_A}{K_A + L} + \frac{n_B K_B}{K_B + L} \quad (1)$$

where K_{obsd} and K_t are the association rate constants for α-BuTx binding to the AChR in the presence and absence, respectively, of the ligand at concentration L, and n_A and n_B are the relative fractions of binding sites for the ligand with dissociation constants of K_A and K_B, respectively. The sum of n_A and n_B was assumed to be 1, and the values for n_A, n_B, K_A, and K_B were obtained by nonlinear regression, using a program written by Dr. James Roberts of the Department of Obstetrics, Gynecology and Reproductive Endocrinology, University of California, San Francisco, and generously made available to us by him. The total amount of receptor was determined in a 3-h incubation with labeled toxin alone.

Table I: Characteristics of mAbs That Block α-BuTx Binding to the AChR

fusion	method of immunization	mAB	isotype	% max inhibition of α-BuTx binding
1	lipopolysaccharide method	A1	IgA	44
2	cyclophosphamide method	B1	IgG	43
		B2	IgG_{2b}	39
		C1	IgG_{2b}	98
		C2	IgG_3	97
3	cyclophosphamide method	A2	IgG_{2b}	46
		43	IgG_1	41
		A4	IgG_{2b}	41
		A5	IgG_1	42
		A5	IgG_1	42
		A6	IgG_3	41
		B3	IgG_{2b}	46
		B4	IgG_{2b}	48

Results

Selection of Hybridomas. Antibodies produced by hybridomas from three fusions were screened in a solid-phase assay to detect those that bound to solubilized *Torpedo* AChR but did not bind to toxin–receptor complex (see Methods). Twelve hybridomas that were positive by this criterion were selected and successfully cloned. The isotypes of the antibodies produced by these hybridomas are listed in Table I. All bind to purified, unreduced AChR with dissociation constants in the range of 10^{-9}–10^{-10} M.

mAbs Distinguish Two Types of Toxin-Binding Sites on the AChR. The antibodies showed differing abilities to block binding of ^{125}I-α-BuTx as measured by a DEAE filter assay. Two of the antibodies were able to block toxin binding completely; the other 10, however, inhibited toxin binding only to a level of about 50%, even at the highest antibody concentrations tested (Figure 1). Antibodies in the latter class were then tested in pairs to determine whether their inhibitory effects were additive. On the basis of these results, the antibodies could be divided into two mutually exclusive groups such that the combination of two antibodies from the same group did not give an inhibition greater than 50%, but two

Table II: Binding of ^{125}I-AChR to Solid-Phase Antibodies after Preincubation with A, B, or C mAbs or with α-Bungarotoxin[a]

	immobilized mAb											
competing mAb	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	C1	C2
A1	−	−	−	−	−	−	+++	+++	+++	+++	+	++
A2	−	−	−	−	−	−	+++	+++	+++	+++	+	++
A3	±	−	−	−	±	−	+++	+++	+++	+++	+	++
A4	−	−	−	−	−	−	+++	++	+++	+++	+	++
A5	−	−	−	−	−	−	+++	++	+++	+++	+	++
A6	−	−	−	−	−	−	+++	++	+++	+++	+	++
B1	+++	+++	+++	+++	+++	+++	−	−	−	−	+++	++
B2	+++	+++	+++	+++	+++	+++	−	−	−	−	+++	++
B3	+++	+++	+++	+++	+++	+++	−	−	−	−	+++	++
B4	+++	+++	+++	+++	+++	+++	−	−	−	−	+++	++
C2	−	−	−	−	−	−	++	+	++	+	−	−
α-BuTx	−	−	−	−	−	−	−	−	−	−	−	−

[a] Reduced and alkylated ^{125}I-AChR was incubated with mAbs (1/50 dilution of ascites fluid) or α-BuTx and then with a solid-phase antibody in microtiter plates as described under Methods. Values were expressed in each case as percent of binding without incubation with blocking mAb or with α-BuTx. (+++) refers to binding that was not significantly different from control values (competing mAb or α-BuTx omitted), (++) to binding that was 50–80% of control values, (+) to binding that was 20–50% of control values, (±) to cases in which 5–15% of control binding was detected, and (−) to binding less than 5%.

antibodies taken from different groups achieved complete inhibition (Figure 1). Thus, the two groups of antibodies appear to recognize different sites. We have arbitarily designated these two groups as A and B, and the antibodies that completely block toxin binding as group C. Antibodies in group C were able to block toxin-binding sites left free by antibodies in either of the other two groups (data not shown); they thus recognize both A and B sites.

When antibody binding to the AChR was measured directly by a solid-phase assay, the same division of the antibodies into three groups could be made. For this experiment, mAbs were attached to microtiter plates, and the ability of other antibodies to block binding of ^{125}I-labeled AChR to the immobilized antibody was determined. The results showed (Table II) that A-type antibodies, but not B, block the binding of labeled AChR to immobilized A antibodies. Conversely, only B-type antibodies blocked binding to immobilized B antibodies. These results show that within each A or B group antibodies compete with each other for binding, and thus interact with neighboring sites on the receptor. A- and B-type antibodies do not compete, however, and thus bind at separate loci. C-type mAbs reduced the binding of labeled AChR to both A and B immobilized mAbs, and therefore bind to both sets of sites. When mAb C1 was the immobilized species, binding of receptor was decreased more effectively by preincubation with A than with B antibodies, suggesting that when attached to the plate, C1 antibodies preferentially recognize the A site (Table II).

Two Antigenically Distinct Toxin-Binding Sites Occur within the Same AChR Oligomer. The experiments described so far demonstrate that the sites recognized by A- and B-type antibodies are distinct. To determine whether the two sites are associated with different molecules or are located on the same receptor, microtiter plates were coated with either A- or B-type antibodies and subsequently incubated with unlabeled, monomeric (9 S) AChR. Toxin binding to the tethered AChR was then determined. The results of these experiments showed (Table III) that tethered receptors retained toxin-binding activity and that in all cases this activity could be decreased by addition of antibodies that belonged to a different group from those used for tethering. In contrast, no inhibition was observed by antibodies of the same group. Thus, sites recognized by both A- and B-type mAbs are associated with the same receptor molecule.

Similar results were found when complexes of AChR and ^{125}I-antibody were tested for their ability to bind to other mAbs attached to microtiter plates. The results demonstrated directly that immobilized A-type antibodies can bind receptor to which B-type antibodies are bound and vice versa. For example (Table IV), AChR–^{125}I-B1 complex bound to solid-phase A- or C-type mAbs, and AChR–^{125}I-A2 or AChR–^{125}I-A6 complexes bound to B- or C-type, but not A-type, mAbs. In these experiments, the formation of the complex itself also showed

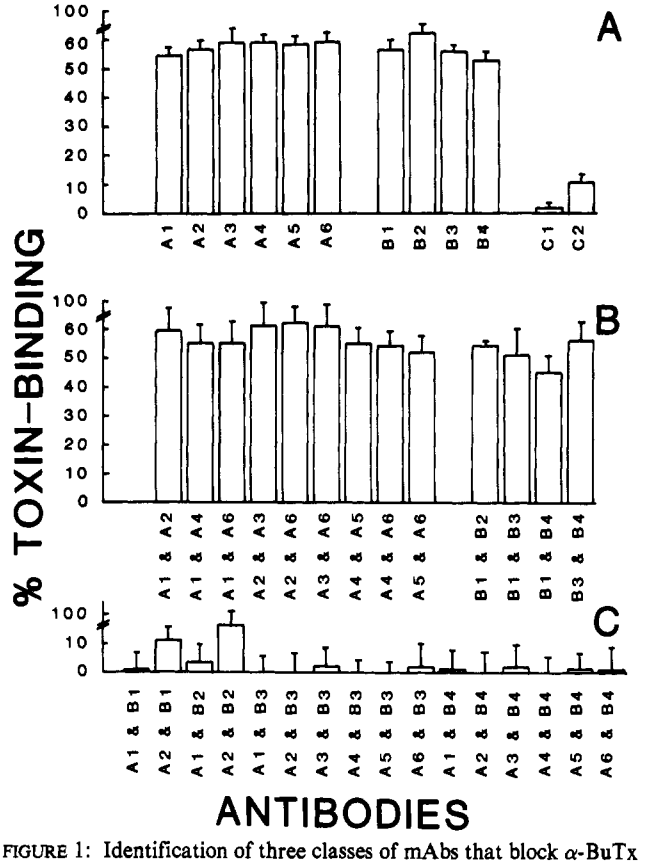

FIGURE 1: Identification of three classes of mAbs that block α-BuTx binding. mAbs were incubated with purified *Torpedo* AChR and then with ^{125}I-α-BuTx as described under Methods. The amount of toxin–receptor complex formed was then measured by using the DEAE filter assay. The values shown represent those for saturating concentrations of each mAb or combination of mAbs. The antibodies were classified according to whether they could block 100% (C) or 50% (A or B) of toxin binding and for A and B antibodies according to their abilities to cause additive inhibition of α-BuTx binding in combination with each other.

Table III: Binding of ^{125}I-α-BuTx to AChR Tethered to Solid-Phase mAbs, after Preincubation with A, B, or C mAbs[a]

	% toxin binding for immobilized mAb									
competing mAbs	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4
A1	92	88	70	88	109	80	25	33	26	30
A2	103	97	74	103	102	93	56	62	58	62
A3	103	108	86	109	95	106	66	69	68	69
A4	99	97	85	104	103	100	57	62	55	62
A5	93	112	91	117	97	105	45	42	44	40
A6	111	108	87	105	104	96	60	66	66	64
B1	11	14	11	16	12	11	91	74	91	90
B2	31	29	34	34	44	32	98	94	95	92
B3	7	10	10	10	12	7	97	88	96	95
B4	9	13	14	15	12	11	97	88	93	92
C1	62	54	49	58	66	54	44	46	42	47
C2	57	44	37	48	51	42	46	44	45	46

[a] Purified AChR was bound to solid-phase mAbs on microtiter plates incubated with A, B, or C mAbs and then with ^{125}I-α-BuTx as described under Methods. Values are expressed as the percent of the value obtained for each immobilized mAb in the absence of blocking mAb.

Table IV: Binding of Complexes Formed between ^{125}I-mAb and AChR to Solid-Phase Antibodies[a]

	immobilized mAb											
mAb–AChR complex	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	C1	C2
^{125}I-B1–AChR	+	+	+	+	+	+	–	–	–	–	+	+
^{125}I-A2–AChR	–	–	–	–	–	–	+	+	+	+	+	+
^{125}I-A6–AChR	–	–	–	–	–	–	+	+	+	+	+	+

[a] Complexes between ^{125}I-mAb and reduced and alkylated AChR were prepared and incubated with immobilized antibodies in microtiter plates as described under Methods. Entries denoted (+) showed strong binding to solid-phase antibodies (>5 times background). Those denoted (–) were not significantly above background. Similar results were obtained in experiments in which dimeric AChR that had not been reduced and alkylated was used.

specificity in competition assays. Thus, the binding of ^{125}I-B1 to the AChR was almost completely inhibited by excess, unlabeled B1, B2, B3, or B4 mAbs but was unaffected by A-type antibodies; conversely, A1, A3, A4, or A5 mAbs, but not B antibodies, competed with iodinated A2 or A6 for binding to the AChR (data not shown).

In the experiments shown in Table II, binding of labeled AChR by immobilized A-, B-, or C-type antibodies was found to be completely inhibited by α-BuTx. Thus, all antibody-binding sites are near toxin sites. Because there are two α-BuTx-binding sites per AChR monomer, and because α-BuTx competes for binding with A-, B-, and C-type antibodies, we conclude that in each monomer there is one binding site each for A- or B-type antibodies and two for C-type antibodies.

Sites Recognized by A- and B-Type Antibodies Bind α-Bungarotoxin with Different Kinetics. The solid-phase assay system was then used to measure the kinetics of binding of α-BuTx to the AChR. Solubilized AChR was tethered to microtiter plates by using a mAb that had no effect on toxin binding as measured by the filter assay (see Methods). Binding to the tethered receptor was measured under pseudo-first-order conditions, using excess ^{125}I-α-BuTx. Typical results are shown in Figure 2. In the absence of antibody, the bound receptor showed complex kinetics, as has been seen by others (Blanchard et al., 1979; Maelicke et al., 1977; Hess et al., 1975; Mihovilovic & Richman, 1984), indicating the presence of more than one site. These data were analyzed as described by Blanchard et al. (1979) by assuming two components for toxin binding, and using the equation:

$$C - C_t = f_1 e^{-k_1(t)} + (1 - f_1)e^{-k_2(t)} \quad (2)$$

where C is the maximum amount of α-BuTx bound, C_t is the amount of α-BuTx bound at time t, k_1 is the pseudo-first-order rate constant for the slow kinetic company, k_2 is the pseudo-first-order constant for the fast kinetic constant, and f_1 is the fraction of the total α-BuTx binding sites that are kinetically slow. The data were fit to the equation by nonlinear regression

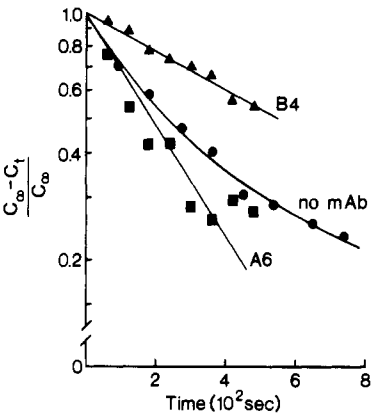

FIGURE 2: Kinetics of binding of α-BuTx to AChR and to AChR–mAb complexes. Purified AChR was tethered to the wells of microtiter plates as described under Methods. Following incubation in some cases with an A or B mAb, the AChR was then incubated with excess ^{125}I-α-BuTx under pseudo-first-order conditions as described under Methods. C represents toxin bound after 120 min of incubation. (●) are the results obtained with AChR alone; (■) with AChR complexed to mAb A6; and (▲) with AChR complexed to mAb B4. The drawn curves represent those obtained by nonlinear regression analysis with eq 2. For the curve with AChR alone, $f_1 = 0.49$, and the second-order rate constants derived from k_1 and k_2 are 1.1×10^5 and 6.0×10^5 M^{-1} s^{-1}, respectively. The values of f_1 for the AChR–A6 and AChR–B4 complexes are 1.0 and 1.02, respectively, and the single second-order rate constants in each case are 3.6×10^5 M^{-1} s^{-1} for the AChR–A6 complex and 1.3×10^5 M^{-1} s^{-1} for the AChR–B4 complex. The difference between the rate constants in the experiment shown is smaller than the difference between the average values given in the text.

as described under Methods. Average values for three separate experiments were $f_1 = 0.59 \pm 0.11$, $k_1 = (0.98 \pm 0.19) \times 10^5$ M^{-1} s^{-1}, and $k_2 = (9.3 \pm 2.4) \times 10^5$ M^{-1} s^{-1} (mean ± SEM, $n = 3$). The values for the kinetic constants are similar to those seen by others (Blanchard et al., 1979; Mihovilovic & Richman, 1984), and the value for f_1 is consistent with a model in which there are two distinct toxin-binding sites on each 9S

Table V: Inhibition of Binding of ^{125}I-AChR to Solid-Phase Antibodies by Cholinergic Ligands: Concentration for 50% Inhibition (M)[a]

mAb	carbamylcholine	*d*-tubocurarine	decamethonium	hexamethonium	pancuronium
A1	3.5×10^{-3}	1.1×10^{-5}	–	–	–
A2	3.6×10^{-4}	2.8×10^{-6}	–	–	–
A3	2.5×10^{-4}	10^{-6}	–	–	–
A4	1.4×10^{-4}	1.2×10^{-6}	$>10^{-3}$	–	5×10^{-8}
A5	1.2×10^{-4}	2.8×10^{-6}	–	–	–
A6	1.8×10^{-3}	1.5×10^{-6}	$>10^{-2}$	–	–
B1	4.5×10^{-3}	1.4×10^{-4}	$>10^{-2}$	–	$>10^{-4}$
B2	–	$\gg 5 \times 10^{-4}$	–	–	$>10^{-4}$
B3	2.8×10^{-3}	10^{-4}	–	–	$>10^{-4}$
B4	3.4×10^{-4}	10^{-4}	2×10^{-3}	–	2×10^{-5}
C1	7.1×10^{-3}	2.2×10^{-6}	1.3×10^{-4}	5.6×10^{-3}	6.3×10^{-8}
C2	5×10^{-3}	1.5×10^{-5}	5.5×10^{-4}	10^{-2}	3.2×10^{-6}

[a] Binding experiments were carried out by incubating ^{125}I-AChR with solid-phase antibodies as described under Methods. Seven concentrations of each ligand were tested over a concentration range of 4 orders of magnitude, and the values for 50% inhibition were extrapolated from the resulting curves. The symbol (–) denotes less than 30% inhibition at the highest ligand concentration (usually 10^{-4} M) tested.

receptor molecule with intrinsically different rates of toxin binding (see Discussion).

Incubation of the tethered receptor with either A- or B-type antibodies altered the rate of toxin binding, but in opposite ways: the reaction rate was increased by the prior binding of A antibodies and decreased by B antibodies. The results suggest that each of the antibody types blocked one of the kinetically distinct components of toxin binding. An experiment with two of the antibodies is shown in Figure 2. In this experiment, incubation with mAb B4 appeared to remove the fast component of toxin binding to yield a single slow component, and incubation with mAb A6 decreased the proportion of the kinetically slow component to give a curve dominated by the fast component of toxin binding. Analysis of the data using eq 2 showed in each case that the data in the presence of the mAb were better fit by a single component than by two components. Furthermore, the kinetic constant of the single site was in each case similar to one of the two constants obtained in the absence of mAb (see legend to Figure 2). Qualitatively similar results were found for other A (A2) and B (B1, B3) antibodies; e.g., rates of toxin binding were faster after incubation with A antibody and slower after incubation with B antibody. In these cases, the reactions in the presence of the antibodies were kinetically more complex, possibly because of a failure to achieve complete inhibition at the relevant site. All of these results are most easily explained by a model in which A antibodies block toxin binding to the kinetically slow component and B antibodies block binding to the kinetically fast component.

Cholinergic Ligands Interfere with mAb Binding to the AChR. Because antibody binding was blocked by α-BuTx, we examined the ability of other nicotinic cholinergic ligands to block binding of the mAbs. Each of the cholinergic ligands that we tested affected the binding of one or more groups of antibodies, but different patterns were observed for different ligands (Tables V and VI). With the exception of mAb B2, binding of all of the mAbs to the AChR was inhibited by the cholinergic agonist carbamylcholine and by the antagonist *d*-tubocurarine (Table V). Carbamylcholine was effective in approximately equal concentrations for all mAbs, while lower concentrations of dTC were required to give equivalent inhibition of binding of A-type than B-type antibodies. The two C-type antibodies were also inhibited by relatively low concentrations of dTC. These results are consistent with those of Neubig and Cohen (1979), who examined the binding of radiolabeled ligands directly and found sites with two affinities for dTC and only one with affinity for carbamylcholine [however, see Prinz and Maelicke (1983)].

Table VI: Inhibition of ^{125}I-AChR Binding to Solid-Phase mAbs by 5 μM α-Conotonin GI[a]

mAb	inhibition	mAb	inhibition
A1	+	B1	++
A2	–	B2	–
A3	–	B3	++
A4	++	B4	++
A5	–		
A6	–	C1	++
		C2	+

[a] Binding of ^{125}I-AChR to immobilized mAbs was determined by a solid-phase assay as described under Methods. Plates to which goat anti-mouse immunoglobulin had been attached were incubated with hybridoma supernatant, followed by ^{125}I-AChR (5.5 nM in α-BuTx sites) that had been incubated for 1 h with the indicated concentration of α-conotoxin GI. (–) refers to binding that was greater than 80% of control values obtained in the absence of α-conotoxin; (+) refers to binding that was between 20% and 80% of control values; (++) refers to binding that was less than 20% of control values.

In contrast to the results obtained with dTC and carbamylcholine, the bisonium ligands tested, decamethonium and hexamethonium, were effective inhibitors only of the C-type antibodies and had no effect on A or B antibodies. A dramatic difference in the effects of A- or B-type antibodies was seen with pancuronium, which blocked binding of all B-type antibodies at millimolar concentrations and was ineffective with all A-type antibodies, except A4, whose binding was blocked at less than micromolar concentrations (Table V).

We also examined the effect of a small peptide toxin, α-conotoxin GI, whose effect on the AChR has been shown to resemble that of α-BuTx (Olivera et al., 1985). α-Conotoxin at micromolar concentrations substantially inhibited the binding of both C mAbs and of some, but not all, A and B mAbs (Table VI). Only the binding of mAbs A1 and A4 was affected by the conotoxin, while the toxin decreased the binding of all B antibodies but B2.

Taken together, the results of these inhibition studies suggest that all of the mAbs that we have isolated, with the possible exception of B2, bind to epitopes that are affected by the binding of cholinergic ligands. The data also show that for both A and B types, antibodies within each group recognize diverse epitopes. For example, A4 binding is inhibited by pancuronium, but this antagonist does not inhibit the binding of any other A-type antibodies, and B2 binding differs from that of other B-type antibodies in not being blocked by carbamylcholine or by α-conotoxin.

A- and B-Type Antibodies Bind to Pharmacologically Distinct Toxin-Binding Sites. The results with cholinergic ligands suggest that A- and B-type antibodies recognize sites

Table VII: Effect of A and B mAbs on Antagonist Binding[a]

	high-affinity site		low-affinity site	
	K_D ($\times 10^{-9}$ M)	fraction	K_D ($\times 10^{-6}$ M)	fraction
	(A) dTC			
without mAb	68 ± 3	0.23	4.6 ± 0.3	0.77
+B3	151 ± 11	1.0		
+A6			5.0 ± 0.2	1.0
+A1			5.4 ± 0.3	1.0
	(B) Pancuronium			
without mAb	1.9 ± 1.0	0.21	9.3 ± 0.8	0.79
+B3	2.0 ± 0.2	1.0		
+A mAbs[b]			8.8 ± 1.0	1.0

[a] The decrease in the initial rate of binding of α-BuTx to the *Torpedo* AChR caused by various concentrations of the antagonists dTC and pancuronium was used to estimate binding constants for these antagonists. Measurements in the presence and absence of A and B mAbs were carried out as described under Methods. Values are expressed as the mean ± the SEM. [b] The values from two experiments, one made with A6 and the other A1, were pooled for this estimate.

with different pharmacological properties. Previous studies have established that several cholinergic ligands bind to the *Torpedo* and muscle receptors with two different affinities (Neubig & Cohen, 1979; Weiland & Taylor, 1979; Sine & Taylor, 1981 Prinz & Maelicke, 1983; Gu et al., 1985; Hamilton et al., 1985; Covarrubias et al., 1986).

To investigate the correspondence between the sites with different affinities for cholinergic ligands and the sites recognized by the mAbs, we used a solid-phase assay system in which the binding of a cholinergic antagonist, either dTC or pancuronium, was measured by its effect on the initial rate of binding of α-BuTx. Both antagonists gave biphasic curves in this assay, suggesting that two sites were involved (Figure 3). Analysis by a two-site binding expression gave a good fit to the data. In the case of both antagonists, however, there was apparently a larger proportion of low-affinity than of high-affinity sites (Table VII). This disproportion must arise in part from the method of assay. If the two pharmacologically distinct sites have intrinsically different rates of toxin binding, experiments performed under initial rate conditions will favor the kinetically faster site. In our experiments, the site with low affinity for antagonists predominated, suggesting that it corresponds to the kinetically faster site. The ratio of low-affinity to high-affinity sites was approximately 4 to 1 (Table VII). This is in fair agreement with the value expected, based on the relative values of the rate constants for toxin binding (9.5 to 1), and the fact that there may be a slight preponderance in our AChR preparations of the kinetically slow site (see above).

When the antagonist inhibition experiments were repeated after the addition of A- or B-type antibodies, the biphasic curves were converted to curves that were best fit by assuming a single binding site (Figure 3). In each case, the B antibody removed the low-affinity site, and the A antibodies removed the high-affinity site. The binding constant of the remaining site was changed little, if at all (Table VII). The results of the antibody experiments are thus consistent with the conclusions of the previous experiments in showing that the A antibodies remove the kinetically faster site and the B antibodies remove the slower site.

Discussion

The experiments reported here establish the specificity of two classes of mAbs for the two toxin-binding sites of the AChR and describe the kinetic and pharmacological properties of these two sites. Additon and competition experiments with combinations of antibodies defined two separate toxin-binding

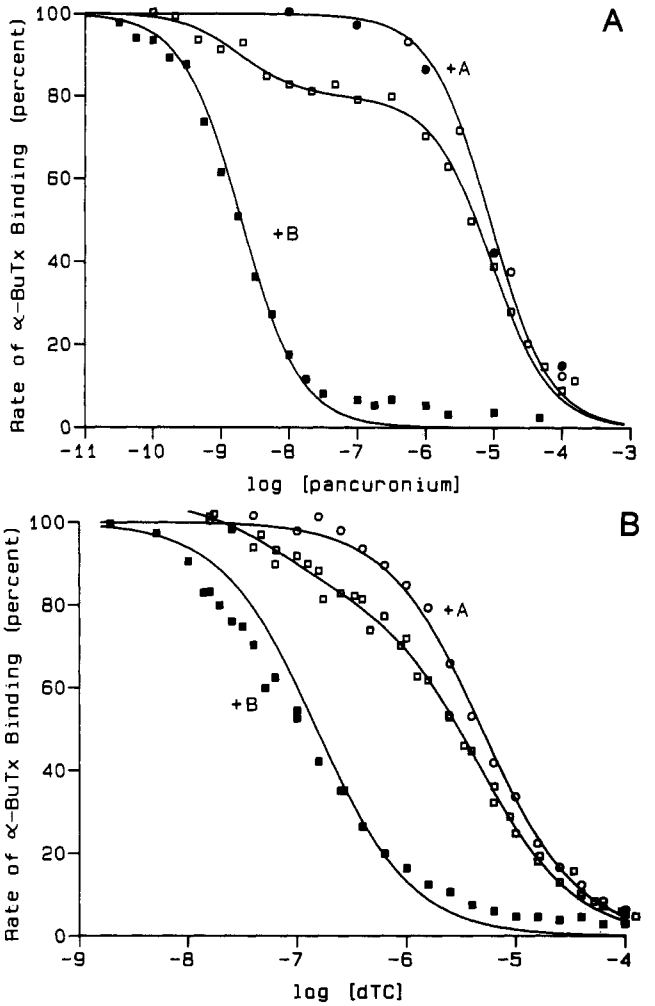

FIGURE 3: Inhibition of toxin binding to AChR and AChR–mAb complexes by dTC and pancuronium. Purified *Torpedo* AChR was tethered to the wells of microtiter plates, as described under Methods. After incubation in some cases with an A or B mAb, the effect of various concentrations of (A) dTC or (B) pancuronium on the initial rate of binding of α-BuTx to the receptor was determined as described under Methods, and curves were drawn according to the values obtained.

sites. We call mAbs that bind near one of these sites group A antibodies, those that bind near the other group B antibodies, and those that recognize both group C antibodies. Since ternary complexes of receptor with both A and B mAbs can be formed, both A and B toxin-binding sites must be on the same AChR. These two sites are presumably associated with each of the two α subunits of the AChR.

We then attempted to relate the sites defined by the antibodies to the components of toxin and ligand binding identified by kinetic or equilibrium binding experiments. In past studies of toxin or ligand binding (Neubig & Cohen, 1979; Sine & Taylor, 1981; Blanchard et al., 1979; Hess et al., 1975; Maelicke et al., 1977; Mihovilovic & Richman, 1984), it has often been difficult to distinguish between binding to two independent sites with intrinsically different properties, and binding to identical sites that show cooperativity. In our experiment, incubation with antibodies in each case selectively removed one of the components, with little change in the properties of the remaining component (Figures 2 and 3; Table VII). These results are most simply explained by assuming that the antibodies recognize two separate sites with differing immunological and pharmacological properties (Table VIII). One site is recognized by A and C antibodies, reacts slowly with toxin, and binds the antagonists dTC and pancuronium

Table VIII: Pharmacological and Kinetic Properties of A and B Sites[a]

property	toxin binding site A	B
binding of mAb	A, C	B, C
kinetics of binding α-BuTx	slow	fast
affinity for dTC		
by inhibition of binding mAb	high	low
by mAb block of toxin binding	high	low
affinity for pancuronium		
by inhibition of binding of mAb	high	low
by mAb block of toxin binding	high	low

[a] A summary of the properties of the two toxin-binding sites of the *Torpedo* AChR. This summary is consistent with the properties of mAbs inhibiting toxin binding that have been described by Fulpius (1980), Mochly-Rosen and Fuchs (1981), Watters and Maelicke (1983), Mihovilovic and Richman (1984), and Whiting et al. (1985).

with high affinity. The other is specifically recognized by B and C antibodies, reacts rapidly with toxin, and binds the antagonists with low affinity. The effects of cholinergic ligands on antibody binding also fit this scheme. Lower concentrations of dTC are required to block the binding of A-type antibodies than of B-type antibodies.

The distinctive immunological and pharmacological properties that we have ascribed to the two toxin-binding sites of the *Torpedo* AChR are also consistent with the results of others. On the basis of the pharmacological properties of the sites that they inhibit, we are able tentatively to identify other antibodies described in the literature as corresponding to either A or B types. For instance, the mAb 247G described by Mihovilovic and Richman (1984) selectively removes the slow component of α-BuTx binding and thus presumably corresponds to an A-type antibody, while the mAbs described by Whiting et al. (1985) appear to be B-type antibodies on the basis of their effects on the binding of *d*-tubocurarine (Whiting et al., 1985), of competition experiments with our antibodies (A. Vincent, A. J. Dowding, and Z. W. Hall, unpublished experiments), and of their ability to decrease the initial rate of toxin binding by more than 50% (P. Whiting and A. Vincent, personal communication). The mAb (5.5) isolated by Mochley-Rosen and Fuchs (1981) has characteristics of an A-type mAb: inhibition of its binding by low concentrations of dTC and its complete blockade of α-BuTx binding to AChR–B complexes but not to AChR–A complexes (A. J. Dowding, S. Fuchs, and Z. W. Hall, unpublished experiments). Finally, the mAbs described by Watters and Maelicke (1983) define two antigenically different toxin-binding sites of the *Torpedo* AChR. The pharmacological properties of these sites are unclear, but they presumably correspond to the two sites described here.

Evidence for two toxin-binding sites with distinctive properties was obtained earlier by Damle and Karlin (1978), who concluded that sulfhydryl affinity reagents are bound preferentially at one of the two sites; recent work by Ratnam et al. (1986) suggests that this site may correspond to the A site. Experiments by Hamilton et al. (1985) indicate that the two pharmacologically distinct toxin-binding sites may have different physical locations within the subunit structure of the AChR. The A and B antibodies described here should be useful tools in further experiments aimed at assigning the two sites to specific locations within the *Torpedo* AChR.

The toxin-binding sites of muscle AChRs also have distinctive pharmacological and immunological properties. The AChRs in the mouse muscle cell lines BC3H-1 and C2 have two toxin-binding sites with different affinities for dTC, but unlike those of the *Torpedo* AChR, these sites have similar association rate constants for the toxin-binding reaction (Sine & Taylor, 1981; Gu et al., 1985). We have previously shown that antibodies in a myasthenic serum specifically block toxin binding to one of these sites (Gu et al., 1985). This site has a higher affinity for dTC and thus may correspond to the A site of the *Torpedo* AChR. Unfortunately, none of the mAbs that we have isolated that block toxin binding of the *Torpedo* AChR cross-reacts with rodent muscle AChR; moreover, antibodies in the myasthenic serum do not block toxin binding of *Torpedo* AChR (A. J. Dowding, B. Olwin, and Z. W. Hall, unpublished experiments). Thus, it is not possible to test this idea directly. Given the high degree of conservation between the α subunits of mouse and *Torpedo* receptors (Boulter et al., 1985) and the importance of the toxin-binding site, the failure of all 12 mAbs to cross-react with rodent muscle receptor is surprising.

The binding of each of the mAbs that we isolated, with the exception of B2, is inhibited by one or more cholinergic ligands. Surprisingly, the effect of different ligands on the binding of a single antibody is quite variable. The binding of A1, A2, A3, and A5, for example, is diminished by carbamylcholine and *d*-tubocurarine, but not by decamethonium, hexamethonium, or pancuronium, all of which might be expected to share at least part of the same binding site. Recent work suggests that the mechanism underlying the competition between cholinergic ligands and antibodies that block toxin binding may involve allosteric interactions rather than simple competition for the same physical site (Fels et al., 1986). Different allosteric effects exerted by different cholinergic ligands could explain the variable effectiveness of the ligands in blocking antibody binding (Covarrubias et al., 1986).

Within both A and B groups, the antibodies probably bind to contiguous sites, since in each case antibody binding is blocked by other mAbs in the same group. The effects of a single cholinergic ligand on the binding of different antibodies show, however, that there are diverse epitopes within each group. A48 for instance, was the only A-type antibody whose binding was blocked by pancuronium, and the binding of B2, in contrast to other B mAbs, was not significantly inhibited by either carbamylcholine or *d*-tubocurarine. Antibody A1 was also distinguished from A2, A3, A5, and A6 by its inhibition by α-conotoxin GI. Similar heterogeneity among mAbs that are apparently specific for a single toxin-binding site has been seen by others (Watters & Maelicke, 1983; Whiting et al., 1985; Mihovilovic & Richman, 1987) and has been the basis of schemes that divide the toxin-binding site into discrete subregions with different pharmacological specificities. Experiments with different photoaffinity derivatives of α-neurotoxins demonstrate that the toxin occupies two large and complex areas on the receptor surface (Tsetlin, 1983). Our antibodies presumably recognize different epitopes within each of these areas.

If A- and B-type antibodies do distingiush the two different toxin-binding sites on the AChR, what do they recognize? The two sites are presumably associated with the two α subunits of the intact receptor. So far as is known, there is no difference in the primary sequence of the two α subunits (Raftery et al., 1980; Noda et al., 1982, 1983a,b; Mishina et al., 1984; Klarsfield et al., 1984).

We suggested in an earlier series of experiments that immunological differences in the two toxin-binding sites might arise from differences in glycosylation (Hall et al., 1983). This interpretation was based on the incorrect assumption that the antibodies in a particular myasthenic serum were acting in an

identical way on both *Torpedo* and muscle receptors and has proved to be wrong (B. Olwin and Z. Hall, unpublished results). Other evidence, however, supports the idea that the carbohydrates associated with the two α subunits may not be identical (Wonnacott et al., 1980; Conti-Tronconi et al., 1984; Ratnam et al., 1986). In addition, Ratnam et al. (1986) have shown that after removal of N-linked sugars, differences remain between proteolytic fragments of the two α subunits that could represent other posttranslational modifications. Such differences could represent part of the immunological specificity seen here. We have attempted to detect intrinsic differences between the two α subunits by immunoblotting. Unfortunately, all of the antibodies that we have isolated bind very weakly or not at all to isolated α subunits after Na-DodSO$_4$ gel electrophoresis. These and similar results by others (Mihovilovic & Richman, 1984; J. Lindstrom, personal communication) suggest that antibodies to the toxin-binding sites often recognize conformational determinants. Such conformational determinants could be different for the two α subunits since each has different neighbors in the oligomeric structure of the AChR. These differences could also account for the immunological and pharmacological distinctions between the two toxin-binding sites. The antibodies that we have described will be useful reagents in further clarifying the structural and functional differences of these two sites.

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Interaction of Synthetic Analogues of Distamycin with Poly(dA-dT): Role of the Conjugated *N*-Methylpyrrole System†

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ABSTRACT: Two synthetic analogues of distamycin (Dst), PPA and PAP, containing a saturated β-alanine moiety substituting for an *N*-methylpyrrole chromophore were studied for their interactions with the double-stranded alternating copolymer poly(dA-dT)·poly(dA-dT) [abbreviated as poly(dA-dT)], with UV absorption and circular dichroism (CD) spectroscopy. The distinctive feature of these analogues is the difference in the extents of extended conjugation due to contiguous pyrrole rings: it decreases in the order Dst > PPA > PAP. Both these analogues bind to poly(dA-dT) in a way similar to Dst, as suggested from the observed red shift in the UV spectra of the ligands upon complexation and the appearance of induced Cotton effects (in the 290–350-nm region) in the CD spectra of the complexes. A comparative study of (i) the spectral features of the complexes between these ligands, Dst and netrospin (Nt) and poly(dA-dT), and (ii) the binding parameters for the association with the polynucleotide suggests that the number and relative positions of the pyrrole moieties influence the spectral features and thermodynamic stabilities of the complexes, and the latter show a progressive decrease in the order Dst > Nt > PPA > PAP. Implications of these results vis-ā-vis the molecular basis of Dst–DNA interaction are discussed.

The oligopeptide antibiotics distamycin (Dst) and netrospin (Nt) (Figure 1) bind specifically to the AT-rich regions of B-DNA structure via the minor groove in a nonintercalative fashion (Zimmer, 1975; Zimmer & Wahnert, 1986). X-ray crystallographic studies (Berman et al., 1979; Kopka et al., 1985), various physicochemical studies in solution (Zimmer & Wahnert, 1986; Wartell et al., 1974; Luck et al., 1974; Kolchinskii et al., 1975), and model building studies (Berman et al., 1979; Wartell et al., 1974; Zasedatelev et al., 1978) have indicated that the above specificity for the B-DNA conformation at AT base pairs originates from the following noncovalent interactions: (a) favorable van der Waals contacts in the minor groove of B-DNA, (b) hydrogen-bond formation between amide NH groups of the ligands and adenine N3 and/or thymine O2 atoms in the minor groove of DNA, and (c) electrostatic interactions between the phosphate backbone of DNA and positively charged terminal groups of the antibiotics. The bow-shaped structures with curved backbones for these molecules facilitate the van der Waals contacts and the hydrogen-bond formation involving the NH groups whose H atoms lie on the concave side of the molecule and point toward the bases A and T in the minor groove (Berman et al., 1979; Kopka et al., 1985). Studies on the interaction of several analogues of Dst and Nt with natural and synthetic DNAs (Zimmer & Wahnert, 1986; Lown et al., 1986) also have supported the above observations.

These studies, however, have not attempted to explain the possible role of the consecutive *N*-methylpyrrole groups (a necessary constituent of the above ligands) in the observed specific binding to DNA. On the other hand, there have also been reports of compounds (Luck et al., 1984; Zimmer et al., 1984) lacking a curved backbone, as in Nt and Dst, or potential hydrogen-bonding sites (Zakrzewska et al., 1983) exhibiting a preference for AT sequences.

In our laboratory, we have undertaken the synthesis and comparative studies of the DNA-binding and antibiotic properties of a series of compounds analogous to Dst and Nt with a view to understanding the conformational and chemical basis of their specific interactions with DNA. In a previous paper we reported the features of interactions of a Dst analogue

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