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Mapping of a Cholinergic Binding Site by Means of Synthetic Peptides, Monoclonal Antibodies, and α-Bungarotoxin†

Bianca M. Conti-Tronconi,*,‡ Fen Tang,‡ Brenda M. Diethelm,‡ Sandra R. Spencer,‡ Sigrid Reinhardt-Maelicke,§ and Alfred Maelicke§

Department of Biochemistry, College of Biological Sciences, University of Minnesota, 1479 Gortner Avenue, St. Paul, Minnesota 55108, and Max-Planck-Institut fur Ernahrungsphysiologie, 201 Rheinlanddamm, D-4600 Dortmund, West Germany

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ABSTRACT: Previous studies by several laboratories have identified a narrow sequence region of the nicotinic acetylcholine receptor (AChR) α subunit, flanking the cysteinyl residues at positions 192 and 193, as containing major elements of, if not all, the binding site for cholinergic ligands. In the present study, we used a panel of synthetic peptides as representative structural elements of the AChR to investigate whether additional segments of the AChR sequences are able to bind α-bungarotoxin (α-BTX) and several α-BTX-competitive monoclonal antibodies (mAbs). The mAbs used (WF6, WF5, and W2) were raised against native *Torpedo* AChR, specifically recognize the α subunit, and bind to AChR in a mutually exclusive fashion with α-BTX. The binding of WF5 and W2 to *Torpedo* AChR is inhibited by all cholinergic ligands. WF6 competes with agonists, but not with low mol. wt. antagonists, for AChR binding. The synthetic peptides used in this study were approximately 20 residue long, overlapped each other by 4–6 residues, and corresponded to the complete sequence of *Torpedo* AChR α subunit. Also, overlapping peptides, corresponding to the sequence segments of each *Torpedo* AChR subunit homologous to α166–203, were synthesized. α-BTX bound to a peptide containing the sequence α181–200 and also, albeit to a lesser extent, to a peptide containing the sequence α55–74. WF6 bound to α181–200 and to a lesser extent to α55–74 and α134–153. The two other mAbs predominantly bound to α55–74, and to a lesser extent to α181–200. Peptides α181–200 and α55–74 both inhibited binding of ^{125}I-α-BTX to native *Torpedo* AChR. None of the peptides corresponding to sequence segments from other subunits bound α-BTX or WF6, or interfered with their binding. Therefore, the cholinergic binding site is not a single narrow sequence region, but rather two or more discontinuous sequence segments within the N-terminal extracellular region of the AChR α subunit, folded together in the native structure of the receptor, contribute to form a cholinergic binding region. Such a structural arrangement is similar to the "discontinuous epitopes" observed by X-ray diffraction studies of antibody–antigen complexes [reviewed in Davies et al. (1988)].

The nicotinic acetylcholine receptors (AChRs)[1] are complex transmembrane proteins formed by homologous subunits [reviewed in McCarthy et al. (1986), Maelicke (1988), and Lindstrom et al. (1987)] which in peripheral tissues, such as fish electroplax and skeletal muscle, are assembled in a stoichiometry of $\alpha_2\beta\gamma\delta$ (Raftery et al., 1980; Conti-Tronconi et al., 1982). The AChR subunits are structurally related, suggesting that they have evolved from a common ancestor (Raftery et al., (1980), and they form a family of proteins with other ligand-gated ion channels, such as the glycine and the $GABA_A$ receptors, in spite of the fact that the latter are selective for the anion chloride rather than cations like the AChR (Grenningloh et al., 1987; Levitan et al., 1988). Elucidation

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*To whom correspondence should be addressed.
‡University of Minnesota.
§Max-Planck-Institut fur Ernahrungsphysiologie.

[1] Abbreviations: AChR, nicotinic acetylcholine receptor; α-BTX, α-bungarotoxin; α-CTX, α-cobratoxin, α-naja toxin; mAb, monoclonal antibody; IgG, immunoglobulin G; Ag, antigen; MIR, main immunogenic region; ELISA, enzyme-linked immunosorbent asay; SPRIA, solid-phase radioimmunoassay; HPLC, high-pressure liquid chromatography; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium; DDF, *p*-(dimethylamino)benzenediazonium fluoroborate; Tris, tris(hydroxymethyl)aminomethane; BSA, bovine serum albumin; Prot A, protein A; TBS, 10 mM Tris buffer, pH 7.4, containing 140 mM NaCl; TBS–Tween, 10 mM Tris buffer, pH 7.4, containing 140 mM NaCl and 0.1% Tween; HST, 10 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl and 0.5% Tween; PBS, 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl; PBS–Tween, 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl and 0.05% Tween-20; PBS–BSA, 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl and 0.4% BSA.

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of the molecular structure of these receptors is a necessary prerequisite to understand the structural basis for their function and ion selectivity.

Central to the AChR function are the binding sites for cholinergic ligands. Although as many as four or five cholinergic sites may exist on one AChR molecule (Maelicke et al., 1977; Smart et al., 1984; Dunn et al., 1983; Conti-Tronconi & Raftery, 1986), only the α subunits have been shown to contain a cholinergic site, because thay are labeled by cholinergic affinity labels [reviewed in McCarthy et al. (1987) and Maelicke (1988)] and they can specifically bind α-bungarotoxin (α-BTX) even after denaturation (Haggerty & Froehner, 1981; Gershoni et al., 1983) or when expressed in frog oocytes (Mishina et al., 1984).

The α subunits contain two cysteinyl (Cys) residues at positions 192 and 193 [reviewed in McCarthy et al. (1987) and Maelicke (1988)] which are labeled by the cholinergic affinity label [4-(*N*-maleimido)benzyl]trimethylammonium (MBTA) (Kao et al., 1984). Several studies of the binding of ^{125}I-α-BTX to proteolytic, synthetic, or biosynthetic peptides suggested that one narrow-sequence segment, flanking and including Cys_{192} and Cys_{192}, forms the cholinergic site (Wilson et al., 1984; Oblas et al., 1986; Neumann et al., 1986a,b; Gershoni, 1987; Ralston et al., 1987; Gotti et al., 1988; Aronheim et al., 1988; Conti-Tronconi et al., 1988; Wilson & Lentz, 1988). Furthermore, a 20-kDa proteolytic fragment of the α subunit, which starts at residue Ser_{173} and contains $Cys_{192,193}$, is photolabeled by *d*-[^{3}H]tubocurarine (Pedersen et al., 1986). A common feature of most of the studies summarized above is that they used either α-BTX, which in the denatured α subunit may preferentially or exclusively bind the sequence flanking $Cys_{192,193}$ only, or affinity alkylation of AChR treated with disulfide-reducing agents, which by definition will establish only single positions within a binding region. A study of the effect of single-residue substitutions on α-BTX binding revealed that when Cys_{192} or Cys_{193} was mutated α-BTX still bound, although with lower affinity, while mutation of either of two other Cys residues at positions 128 and 142 of the α subunit completely abolished α-BTX binding (Mishina et al., 1985). In another study, MBTA seemed to label Cys_{142} (Cahill & Schmidt, 1984). A recent study employing the photoaffinity cholinergic label *p*-(dimethylamino)benzenediazonium fluoroborate (DDF), which acts as a competitive cholinergic antagonist, showed that several amino acids, located in distinct regions of the α subunit sequence, were labeled in an agonist-protectable manner (Dennis et al., 1988).

In order to confirm the location of a main constituent loop of the cholinergic binding site to the sequence segment containing residues Cys_{192} and Cys_{193}, and to localize other possible accessory loops, we have studied the binding of α-BTX and of three different monoclonal antibodies (mAbs), raised against *Torpedo* AChR and able to compete with α-neurotoxin and other cholinergic ligands (Watters & Maelicke, 1983; Fels et al., 1986), to a panel of synthetic peptides corresponding to the complete *Torpedo* α-subunit sequence [as reported in Noda et al. (1983a)]. The mAbs preferentially recognize different segments of the α-subunit sequence. MAb WF6 binds primarily to a peptide corresponding to the sequence α181–200 in addition to two other peptides, corresponding to the segments α55–74 and α134–153. The two other mAbs recognize primarily a peptide corresponding to the sequence α55–74 and to a lesser extent to peptide α181–200. Direct binding of ^{125}I-α-BTX to the peptides and competition experiments between the different peptides and native *Torpedo* for ^{125}I-α-BTX binding confirmed that both sequence segments α194–200 and α55–74 contribute to form the α-BTX binding site. A summary of preliminary experiments for the studies reported here was presented at the NATO Workshop "Nicotinic Acetylcholine Receptors in The Nervous System", and it is reported in the proceedings of that meeting (Conti-Tronconi et al., 1988).

Materials and Methods

Peptide Synthesis and Characterization. Overlapping peptides 18–21 residues long, corresponding to the complete *Torpedo* α-subunit sequence [as reported by Noda et al. (1983a)], were synthesized according to Houghten (1985). The peptides corresponded to the following sequence segments: Tα1–20, Tα15–33, Tα30–47, Tα43–60, Tα55–74, Tα63–80, Tα75–94, Tα91–110, Tα106–122, Tα118–137, Tα126–145, Tα134–153, Tα150–169, Tα165–184, Tα181–200, Tα197–216, Tα214–234, Tα230–249, Tα246–265, Tα261–280, Tα276–295, Tα291–308, Tα304–322, Tα318–336, Tα332–350, Tα346–364, Tα360–378, Tα374–394, Tα390–409, Tα406–423, Tα420–437. A peptide corresponding to the sequence Tα67–76 repeated twice [T$\alpha(67–76)_2$] was also synthesized. We also synthesized overlapping peptides corresponding to the sequence segments of all *Torpedo* AChR subunits homologous to the sequence α166–203 [as reported in Noda et al. (1983a)]. These peptide corresponded to the following sequence segments: Tα166–184, Tα172–192, Tα184–203, Tβ176–194, Tβ189–208, Tγ175–193, Tγ192–211, Tδ180–199, Tδ198–217.

Peptides are indicated by the letters Tα–δ for the different *Torpedo* AChR subunits, followed by numbers indicating the position on the subunit sequence of the residues included in the peptide. Peptides which did not have any lysine residue within their sequence, including Tα55–74, were synthesized with a lysine residue at the carboxyl terminus, to facilitate coupling if needed. Peptide Tα55–74 was synthesized both with and without an extra lysine at the carboxyl terminus.

Analysis by reverse-phase high-pressure liquid chromatography (HPLC), using a C18 column (Ultrasphere ODS) and a gradient of acetonitrile in 0.1% trifluoroacetic acid in water, consistently revealed a main peak which accounted for 65–85% of the total optical density. By this approach, peptide purity is underestimated, because of the presence of low molecular weight contaminants which absorb at the wavelength used (214 nm). The sequence and purity of some randomly selected peptides were further verified by gas-phase sequencing (Applied Biosystems). The expected sequence was consistently found, and contaminating sequences (shorter homologous peptides where one or more residues are randomly missing along the sequence because of incomplete coupling) were 10–15% or less. The composition of all peptides was determined by amino acid analysis using PTC derivatives of the amino acids released by acid hydrolysis (Heinrickson & Meredith, 1981), which yielded a satisfactory correspondence between experimental and expected values for all peptides.

Monoclonal Antibodies. The production and characterization of the mAbs used in this study (W2, WF5, and WF6) have been described in detail previously (Watters & Maelicke, 1983; Fels et al., 1986). Antibodies were purified from the hybridoma supernatant by precipitation with ammonium sulfate (50% v/v) followed by chromatography on protein-A Sepharose 4B CL (Pharmacia) of the redissolved and desalted pellet. The bound IgG was eluted with 0.15 M NaCl/0.1 M acetic acid into tubes containing 1 M Tris, pH 9.0. The pH of the eluted fractions was adjusted to approximately 7. The fractions containing mAb were pooled, dialyzed against 10 mM

Table I: IgG Subclass, Cross-Reactivity Pattern, and Competition with Cholinergic Ligands for AChR of the mAbs Used in This Study[a]

hybridoma clone	IgG subclass	cross-reactivity with AChR from				competition with cholinergic ligands		
		Electrophorus[b]	rat[c]	chick[d]	*Locusta*[e]	α-NTX	acetylcholine, carbamoylcholine, succinyldicholine	tubocurarine, bismethonium compounds
W2 (2-B4-12)	2a	−	−	−	−	+	+	+
WF5 (N-2-F9)	2b	+	+	+	+	+	+	+
WF6 (XR-6-G10)	2a	+	+	+	−	+	+	−

[a] The data are from Watters and Maelicke (1983). [b] Affinity-purified AChR from *Electrophorus electricus*. [c] Rat myotube membranes. [d] Chick sympathetic ganglion membranes. [e] Head ganglion membranes from *Locusta migratoria*.

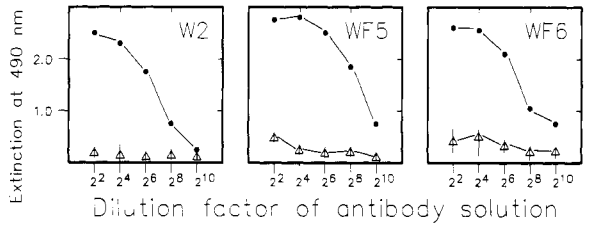

FIGURE 1: AChR subunit specificity of the mAbs used in this study. The different subunits were purified by preparative electrophoresis from SDS-denatured *Torpedo* AChR and used in ELISA experiments to test the specificity of the mAbs. All three mAbs recognized the α subunit (circles) while they did not bind at all to a mixture of the other purified subunits (triangles).

sodium phosphate buffer (pH 7.4)/140 mM NaCl (phosphate-buffered saline, PBS), diluted to the desired optical density (routinely 1.4 units of OD_{280}, i.e., approximately 1 mg/mL), and stored in aliquots at −80 °C.

MAb WF6 binds to the α subunit (Fels et al., 1986). The subunit specificity of the other two mAbs was determined by enzyme-linked immunosorbent assay (ELISA) using *Torpedo* AChR subunits purified by preparative SDS–polyacrylamide gel electrophoresis (Laemmli, 1970). They also recognized only the α subunit (Figure 1). MAbs WF5 and WF6 bind to the AChR molecule with a 1:1 stoichiometry (Fels et al., 1986), which is frequent for mAbs directed against the α subunit (Conti-Tronconi et al., 1981).

Although all these mAbs compete for the binding of α-neurotoxins (Watters & Maelicke, 1983; Fels et al., 1986; see below), their binding is not mutually exclusive. WF6 can inhibit the binding of both other mAbs, while W2 and WF5 only partially inhibit WF6 binding (Watters & Maelicke, 1983). The binding of these mAbs to *Torpedo* AChR is inhibited to different extents by low molecular weight cholinergic agonists and antagonists. MAb WF6 binding is inhibited by cholinergic agonists, not by low molecular weight cholinergic antagonists, while WF5 and W2 are inhibited by both cholinergic agonists and antagonists. Although these mAbs inhibit the binding of α-CTX completely, they inhibit only 50% of the cholinergic agonist binding (Fels et al., 1986; Kuhlmann and Maelicke, unpublished results). Table I summarizes the characteristics of the mAbs and the codes by which they were indicated in previous studies.

Purification, Radiolabeling, and Calibration of ^{125}I-α-BTX. α-BTX was purified from *Bungarus multicinctus* venom (Biotoxins Inc., St. Cloud, FL) according to Clark et al. (1972). Toxin purity was assessed by SDS gel electrophoresis (Laemmli 1970) using an exponential gradient of polyacrylamide (8–20%) and by NH_2-terminal amino acid sequencing using a gas-phase sequenator (Applied Biosystems). Only one peptide band of the expected apparent molecular weight and the known sequence of α-BTX was found. The purified toxin was radiolabeled with ^{125}I using chloramine T (Greenwood et al., 1963), and its specific activity was determined as described in Blanchard et al. (1979).

Preparation of AChR-Rich Torpedo Postsynaptic Membranes. AChR-rich membrane fragments were prepared from *Torpedo californica* electric tissue Elliott et al., 1980) and extracted at pH 11 to remove nonreceptor proteins (Neubig et al., 1979). Upon SDS gel electrophoresis (Laemmli, 1970), these preparations showed only the four AChR subunits as the major protein components. Their specific activity, measured as nanomoles of α-BTX binding sites per milligram of protein by the method of Schmidt and Raftery (1973), was 4–7 nmol/mg of protein (maximum theoretical activity of pure AChR, 7.2 nmol/mg). The AChR-rich membranes were stored at 4 °C and used within 1 week. When needed, the AChR was solubilized by incubating the pH 11 treated membrane fragments in 10 mM sodium phosphate buffer, pH 7.4, containing 1% Triton X-100. The undissolved material was removed by centrifugation.

Inhibition by mAb WF6 of α-BTX Binding to Native Torpedo AChR. AChR-rich membrane fragments or solubilized AChR (25 nM in 10 mM sodium phosphate buffer, pH 7.4) was incubated for 1 h at room temperature with increasing amounts of mAb WF6 or without any antibody. ^{125}I-α-BTX was then added (2 pmol in 50 μL) and incubated for 1 h at room temperature; 100-μL aliquots were pipetted onto DEAE-cellulose disks. Control samples for nonspecific ^{125}I-α-BTX binding were preincubated for 2 h at room temperature with 5 μM unlabeled α-BTX prior to addition of ^{125}I-α-BTX. The disks were washed in 10 mM sodium phosphate buffer, with or without 0.1% Triton X-100, and counted in a Beckman 5000 γ counter. The amount of radioactivity nonspecifically bound was subtracted from the total ^{125}I-α-BTX binding.

Mapping of ^{125}I-α-BTX Binding by Dot Blot Assay. Aliquots (0.5–1 μL) of solutions of the different peptides (1 mg/mL in 10 mM sodium phosphate buffer, pH 7) were spotted onto nitrocellulose strips and allowed to dry. Before usage, the strips were incubated for 45 min at room temperature in 1 mL of 5 mg/mL cytochrome *c* (Sigma) in TBS–Tween. This step reduced unspecific binding of ^{125}I-α-BTX, because cytochrome *c* has charge properties similar to α-BTX. ^{125}I-α-BTX (2–4 μM for mapping the sequence segments able to bind ^{125}I-α-BTX, 0.05–15 μM for determination of the dose dependence of binding) was added. After 6–14 h of incubation at 4 °C or 2 h of incubation at room temperature, the strips were washed 5 times for 30 s with 10 mM Tris buffer, pH 7.4, containing 140 mM NaCl and 0.1% Tween (TBS–Tween) and dried. The strips were autoradiographed and the resulting autoradiograms scanned for optical density in a Bio-Rad 620 densitometer, or the single dots were cut out and counted in a Beckman γ counter. As "blank values" were used either the binding in the presence of an excess (100 μM) of unlabeled toxin (see below) or the binding obtained for peptides corresponding to the sequence α214–437, which general consensus indicates to be either cytoplasmic or transmembrane but not extracellular. Because they also greatly vary in their amino acid sequences, charges, and hydrophilicity, their average

binding is a good estimate of the possible unspecific binding to unrelated sequences. Nonspecific binding either in the presence of excess unlabeled toxin or to control peptides was of similar magnitude, and in both cases increased linearly with the concentration of ^{125}I-α-BTX (Figure 4). The affinity of ^{125}I-α-BTX binding to peptide Tα181–200 was deduced by Scatchard analysis of the concentration dependence curves using the program EBDA (McPherson, 1983).

The ability of unlabeled α-BTX to block ^{125}I-α-BTX binding was assessed by preincubating overnight at 4 °C the peptide-dotted strip with or without 80–100 μM α-BTX in TBS–Tween containing 2 mg/mL cytochrome *c*; 4 μM ^{125}I-α-BTX was then added to both strips and incubated for 2 h at room temperature. The strips were washed 5 times for 30 s with TBS–Tween, dried, and autoradiographed, or the single dots were cut out, and the bound radioactivity was counted in a Beckman γ counter.

Competition between Peptides and Native AChR for ^{125}I-α-BTX Binding. ^{125}I-α-BTX (2 pmol) was incubated for at least 4 h at 4 °C with a 200 μM sample of the peptide to be tested or without any peptide, in PBS, in a final volume of 100 μL. One picomole of membrane-bound AChR in 5 μL of PBS was added to each sample. The samples were incubated for 5 min at room temperature and centrifuged for 1 h at 13000 rpm in an SH-MT Sorvall rotor. The supernatants were discarded, and the AChR-rich membrane fragment was washed again by centrifugation as described above. The amount of ^{125}I-α-BTX bound to the membranes was counted in a Beckman γ 5500 counter.

Mapping of mAb Binding Sites by ELISA and Solid-Phase Radioimmunoassay (SPRIA). Binding of the mAbs to the peptides was assayed by ELISA as previously described (Tzartos et al., 1988) with the following modifications. The mAbs were diluted in PBS containing 0.4% BSA (PBS–BSA) and incubated for 2.5 h at room temperature. After incubation with the second antibody (rabbit anti-mouse IgG), the wells were incubated for 150 min at room temperature with 50 μL/well of a 1:1500 urease-labeled sheep anti-rabbit IgG (Sigma), diluted in PBS–BSA, and then washed 3 times with PBS–Tween. Urease 590 substrate solution (Sigma) was adjusted to pH 4.8 with either NaOH or HCl, and 100 μL was added to each well. The plates were incubated at room temperature for 15–60 min. The reaction was stopped by adding 10 μL/well of a 1% solution of thimerosal (Sigma), and the plates were read at 590 nm in a Titertek ELISA reader. As blank values were considered either the reading of wells without any peptide, or without the mAb, or the average of the values of optical density obtained for peptides corresponding to the segments of the sequence α214–437, for reasons discussed above. In all cases, the blank values obtained were of the same magnitude. Binding of the mAbs to the peptides was also studied by SPRIA as described by Wilson and Lenz (1988).

Mapping of mAb Binding Sites by Dot Blot Assay. Aliquots (0.5–1 μL) of solutions of the different peptides (0.1–1 mg/mL in 10 mM sodium phosphate buffer, pH 7) were spotted onto nitrocellulose strips and allowed to dry. Before usage, the strips were washed twice for 3 min with 3 mL/strip of TBS–Tween. Diluted antibody solution in TBS–Tween (0.6 mL) was added to each strip and incubated with gentle agitation for 2 h at room temperature. The strips were washed once with 3 mL/strip of 10 mM Tris-HCl buffer, pH 7.4, containing 500 mM NaCl and 0.5% Tween (high-salt Tris buffer, HST) and twice for 5 min with 3 mL/strip of TBS–Tween. The presence of bound antibody was revealed by the use of protein A (Prot A), which directly binds to these mAbs (Watters & Maelicke, 1983; Fels et al., 1986). The strips were incubated with 1.2 mL of ^{125}I-Prot A solution (1.2×10^6 cpm/mL), labeled with chloramine T (Greenwood et al., 1963) in TBS–Tween containing 1 mg/mL BSA, for 3 h at room temperature, washed 5 times with TBS–Tween, dried, and autoradiographed. Blank strips were processed as described above but omitting the mAb.

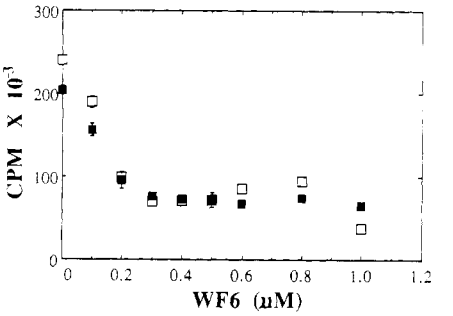

FIGURE 2: Inhibitor effect of mAb WF6 on the binding of ^{125}I-α-BTX to native *Torpedo* AChR. Membrane-bound (closed squares) and Triton X-100 solubilized (open squares) AChR was used. With either preparation, preincubation with increasing amounts of WF6 progressively inhibited ^{125}I-α-BTX binding, up to 81 ± 2.5% (n = 4) of the total specific binding obtained in the absence of antibody.

Reduction/Alkylation or Oxidation of Peptides Tα181–200. A 350 μM solution of the peptide in 10 mM sodium phosphate buffer, pH 8, was used. For reduction and alkylation, 3.5 mM dithiothreitol was added to 0.5 mL of peptide solution, and the mixture was incubated for 1 h at room temperature under nitrogen. Iodoacetamide was added to a final concentration of 10.5 mM and incubated for 5 min at room temperature. The mixture was dialyzed against 10 mM sodium phosphate buffer, pH 7, using dialysis tubing with a molecular weight cutoff of 1000 (Spectraphor 1000). For oxidation of the peptide, 1.75 mM iodosobenzoic acid was added to 0.5 mL of peptide solution. The mixture was incubated for 1 h at room temperature under nitrogen and dialyzed as described above. The sulfhydryl groups present in the reduced/alkylated and in the oxidized samples, as well as in the original solution of native peptide, were assayed by using their ability to react with 5,5′-dithiobis(2-nitrobenzoic acid) as described by Ellman et al. (1981). The molar extinction coefficient at 420 nm of the colored product obtained is 13600 (Ellman et al., 1981).

RESULTS

α-BTX and mAbs W2, WF5, and WF6 act as cholinergic antagonists of *Torpedo* AChR (Watters & Maelicke, 1983; Fels et al., 1986). Competition between these mAbs and α-neurotoxins has been shown so far for α-CTX only. To further verify that α-CTX and α-BTX bind to the same area of the AChR surface, we investigated whether there is also competition for AChR binding between mAb WF6 and ^{125}I-α-BTX. Both membrane-bound and Triton X-100 solubilized AChRs were used. With either preparation, preincubation with increasing amounts of WF6 progressively inhibited ^{125}I-α-BGT binding, up to 81 ± 2.5% (n = 4) of the specific binding obtained in the absence of antibody (Figure 2). A residual small amount of specifically bound ^{125}I-α-BTX (approximately 19%) in the presence of WF6 was consistently found.

Binding of ^{125}I-α-BTX to Synthetic Peptides. In dot blot assay, ^{125}I-α-BTX (2–4 μM) consistently bound to peptide Tα181–200. In most experiments, peptide Tα55–74 was also recognized, although to a more limited extent (Figure 3). Preincubation with an excess of unlabeled α-BTX inhibited

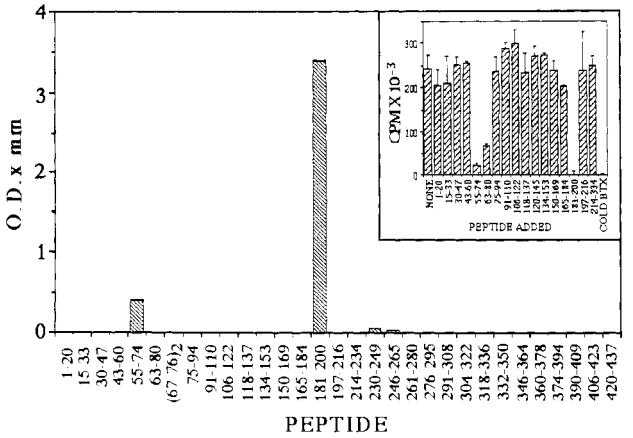

FIGURE 3: Dot blot assays of the binding of ^{125}I-α-BTX (4 μM) to synthetic peptides corresponding to the complete sequence of *Torpedo* α subunit. Direct binding: Peptide Tα181–200 was consistently, strongly recognized. In most experiments, like in the one illustrated here, peptide Tα55–74 was also recognized, although to a more limited extent. In the inset (competition binding), the results of an experiment assessing the ability of peptides corresponding to segment 1–234 of the α subunit (forming the putative extracellular portion of the α subunit plus the first putative transmembrane segment) to compete with native *Torpedo* AChR for ^{125}I-α-BTX binding are reported. The columns indicate the amount of ^{125}I-α-BTX bound by membrane-bound AChR in the absence of any peptide (column "none"), in the presence of an excess of unlabeled α-BTX (column "cold BTX"), and in the presence of 0.5 mg/mL sample of the synthetic peptide indicated under the abscissa. Peptide Tα181–200 completely inhibited the specific ^{125}I-α-BTX binding. Peptide Tα55–74 and the overlapping peptide Tα63–80 also significantly inhibited ^{125}I-α-BTX binding. See text for further details.

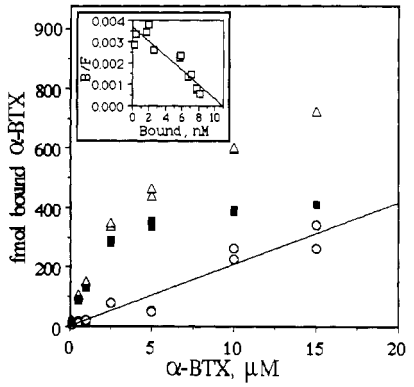

FIGURE 4: Dose dependence of ^{125}I-α-BTX binding to peptide Tα181–200, investigated by dot blot assay. (△) Total binding; (○) nonspecific binding; (■) specific binding as obtained by subtraction of nonspecific binding from total binding. Scatchard analysis of the data suggested the presence of a single population of binding sites, with an apparent K_d of 2.96 μM (inset).

up to 77% of ^{125}I-α-BTX binding.

The dose dependence of ^{125}I-α-BTX binding to peptide Tα181–200 is shown in Figure 4. Scatchard analysis of the data indicated the presence of a single population of binding sites (Figure 4, inset), with an apparent K_d of 2.8 ± 1.44 μM (n = 3). Because of the solid-phase nature of the assay, this value should be considered an upper limit, and the actual K_d could be considerably lower. The K_d values obtained are within the range of affinities found in other studies for denatured α subunit (Haggerty & Froehener, 1981) and synthetic (Neumann et al., 1986a,b; Gotti et al., 1988) or biosynthetic (Gershoni, 1987) peptides.

The binding of ^{125}I-α-BTX to peptides corresponding to the sequence segments homologous to α166–200 of all four *Torpedo* subunits (see Materials and Methods) was investigated. Only peptide Tα184–203, which largely overlaps Tα181–200,

Table II: Inhibition of ^{125}I-α-BGT Binding to *Torpedo* AChR by Synthetic Peptides (0.5 mg/mL)

	% inhibition by synthetic peptides				
expt	Tα181–200	Tα55–74-(K)	Tα55–74	Tα63–80	Tα(67–76)$_2$
1	100	72	91	72	ND
2	97	81	89	57	ND
3	100	74	93	ND	ND
4	90	64.5	ND	ND	ND
5	99	ND[a]	92	89	77
6	92	ND	82	78	48
	av 96.3 ± 4.3	73 ± 7	89 ± 4.4	74 ± 13	62.5 ± 20

[a] ND, not determined.

consistently and specifically bound ^{125}I-α-BTX.

The sequence segment containing the cysteinyl residues at positions 128 and 142 has been proposed to be part of the binding site for cholinergic ligands. We therefore synthesized a peptide corresponding to the sequence segment Tα126–145 and investigated its ability to bind ^{125}I-α-BTX. Because it has been suggested that the existence of a disulfide bridge between Cys_{128} and Cys_{142} is necessary for α-BTX binding, the peptide was used either without any further treatment or after oxidation or reduction and alkylation of its sulfhydryl groups. Peptide Tα126–145, either in its "native" form or after reduction/alkylation or oxidation, did not bind ^{125}I-α-BTX.

Inhibition by Synthetic Peptides of ^{125}I-α-BTX Binding to Native AChR. Peptides corresponding to the segment 1–234 of the α-subunit sequence (i.e., those forming the putative extracellular portion of the α subunit, plus the first putative transmembrane segment) were tested for their ability to compete with *Torpedo* AChR for ^{125}I-α-BTX binding; 0.5 mg/mL peptide Tα181–200 almost completely inhibited ^{125}I-α-BGT binding (96 ± 4%, n = 6). Peptide Tα55–74 also strongly inhibited the binding (89 ± 4%, n = 5). The peptide corresponding to the sequence Tα55–74 plus an extra lysine residue at the carboxyl terminus [Tα55–74(K)] inhibited to a lesser extent (73 ± 7%, n = 4). The only other peptide which had inhibitory ability was Tα63–80 (74 ± 13%, n = 4) which partially overlaps Tα55–74. We therefore tested the effect of 0.5 mg/mL peptide Tα(67–76)$_2$, which inhibited ^{125}I-α-BTX binding, although to a lesser extent than peptide Tα63–80 (62.5 ± 20%, n = 2). The inset of Figure 3 reports the results of a typical experiment, and Table II summarizes the results obtained in different experiments. Peptide Tα126–145 in its "native" oxidized or reduced/alkylated forms did not inhibit ^{125}I-α-BTX binding.

Identification of Sequence Segments Recognized by mAb WF6. When studying the binding to small synthetic peptides of antibodies raised against the native form of a protein antigen, we rely on the ability of the peptides to assume many low-energy conformations, including conformation(s) similar to the native protein, compatible with antibody binding. When peptides are attached to a solid support, like in dot blots, ELISA, and SPRIA procedures, their freedom to assume certain conformations is reduced, and falsely negative results may ensue. To reduce this possibility, we used both nitrocellulose and polycarbonate as solid supports. Direct binding of mAb WF6 to synthetic peptides corresponding to the complete α-subunit sequence was investigated by all three approaches described under Materials and Methods, i.e., by ELISA, SPRIA, and dot blots assays. Nonspecific binding was determined in all cases by performing the assay in the absence of the monoclonal antibody. In ELISA and SPRIA experiments, it was minimal for all peptides. In dot blot experiments, elevated levels of nonspecific binding were ob-

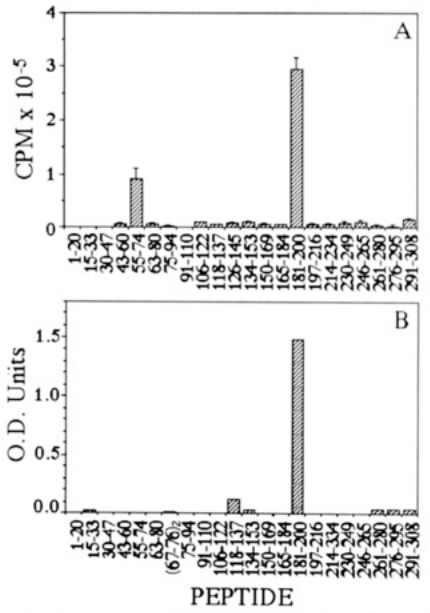

FIGURE 5: Determination by SPRIA (frame A) and by ELISA (frame B) of the binding of mAb WF6 to our panel of synthetic peptides. mAb WF6 strongly recognized peptide Tα181–200. In SPRIA experiments, it also recognized peptide Tα55–74. In some ELISA experiments, like the one illustrated here, it also recognized, although to a much lesser extent, peptide Tα118–137.

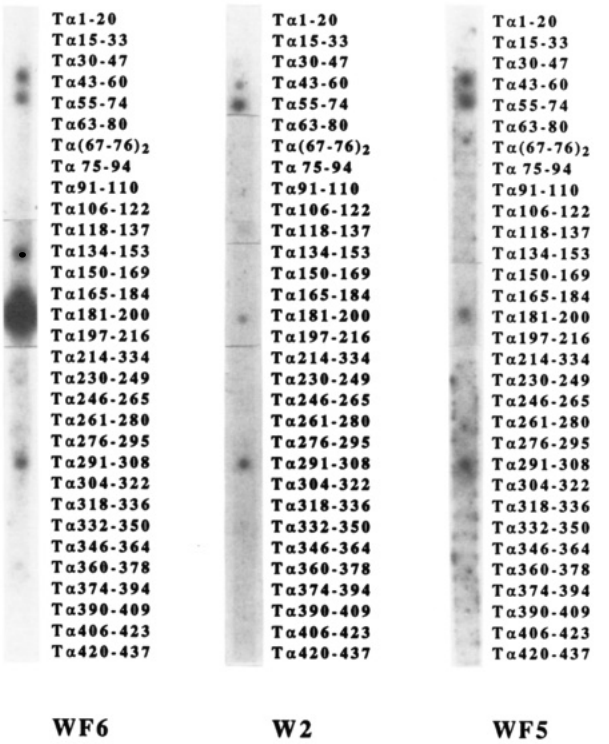

FIGURE 6: Dot blot assays of the binding of mAbs WF6, W2, and WF5 to our panel of synthetic peptides. Binding to peptides Tα43–60 and Tα291–308 was also present in negative controls, in the absence of the relevant mAb, and hence was disregarded as nonspecific. mAb WF6 strongly recognized peptide Tα181–200. It also recognized, although to a lesser extent, Tα55–74, Tα134–153, and Tα165–184, the latter of which partially overlaps Tα181–200. W2 and WF6 clearly recognized Tα55–74, and to a lesser extent Tα181–200.

served for peptides Tα43–60 and Tα291–308.

In both ELISA and SPRIA experiments, mAb WF6 strongly recognized peptide Tα181–200. In SPRIA experiments, it also consistently recognized peptide Tα55–74 (Figure 5A). In ELISA experiments, peptide Tα118–137 was also occasionally recognized (Figure 5B), but the binding to this

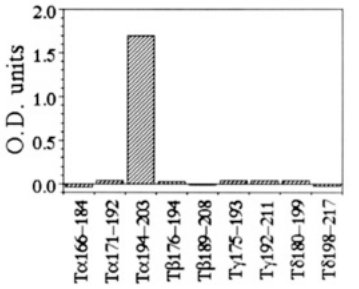

FIGURE 7: ELISA of the binding of mAb WF6 to peptides corresponding to the sequence segments homologous to α166–200 of all four *Torpedo* subunits. Only peptide Tα194–203, which largely overlaps Tα181–200, was recognized.

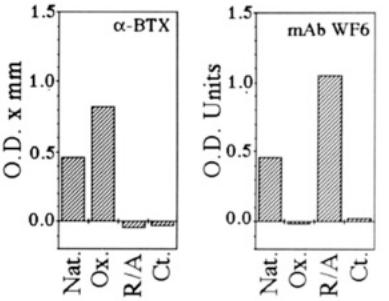

FIGURE 8: Binding of mAb WF6 and of ^{125}I-α-BTX to Tα181–200 in its "native", oxidized, and reduced/alkylated form. WF6 bound significantly better to the reduced/alkylated peptide than to its "native" form, and did not bind at all after oxidation. Reduction and oxidation of Tα181–200 have opposite effects on the binding of ^{125}I-α-BTX; i.e., oxidation improves ^{125}I-α-BTX binding, while after reduction and alkylation ^{125}I-α-BTX does not bind to the peptide to any appreciable amount.

peptide, although specific, was close to the limits of detection by the assay applied. In dot blot assays, in addition to a very strong recognition of peptide Tα181–200, mAb WF6 specifically bound to peptides Tα55–74, Tα134–153, and Tα165–184 (which partially overlaps Tα181–200) (Figure 6). The small amount of binding of WF6 to peptides Tα43–60 and Tα291–308 was similar to negative controls and disregarded as nonspecific (see above).

The binding of mAb WF6 to peptides corresponding to the sequence segments homologous to α166–200 of all four *Torpedo* subunits was studied. Both in ELISA (Figure 7) and in dot blot (not shown) experiments, only peptide Tα184–203, which largely overlaps Tα181–200, was consistently recognized.

Effect of Oxidation and Reduction/Alkylation of Tα181–200 on WF6 Binding. ^{125}I-α-BTX Binding. Stock solutions of Tα181–200, in the absence of reducing or oxidizing agents, have 50–70% of the sulfhydryl groups in the reduced form. After reduction/alkylation or oxidation of two different stocks of Tα181–200, as described under Materials and Methods, only 8 ± 2.5% (n = 2) and 8.1 ± 5.4% (n = 2), respectively, of the cysteine residues had free sulfhydryl groups. WF6 bound significantly better to the reduced/alkylated peptide than to its native form, and did not bind at all after oxidation (Figure 8). Reduction or oxidation of Tα181–200 had opposite consequences on ^{125}I-α-BTX binding; i.e., oxidation increased α-BTX binding, while reduction and alkylation abolished it (Figure 8).

Identification of Sequence Segments Recognized by mAbs WF5 and W2. The binding of mAbs W2 and WF5 was best observed in dot blot assays. W2 and WF5 mAbs specifically and consistently bound to peptide Tα55–74 and, although to a smaller extent, to peptide Tα181–200 (Figure 6). Therefore, their epitopes are formed primarily by residues within the

sequence α55–74. Some nonspecific binding of both W2 and WF5 and of negative controls was observed for peptides Tα43–60 and Tα291–308 (Figure 6).

Discussion

This study indicates that the α-BTX binding site is not a single residue or a single narrow sequence segment (comparable to an "active site") but it rather consists of discontinuous sequence segments of the AChR α subunit, probably folded together in the native structure of the AChR. These segments are the already described sequence segment flanking Cys_{192} and Cys_{193}, the sequence segment α55–74, and possibly also α134–153. Peptides Tα181–200 and Tα55–74 directly bound α-BTX and the anticholinergic site mAbs, and both peptides competitively inhibited ^{125}I-α-BTX binding to native *Torpedo* AChR. Because one mAb (WF6) bound to the sequence α134–153, and possibly to the overlapping peptide α118–134 (Figure 5 and 6), our results do not exclude, and indeed give some support to, the possibility that residues from the sequence segment flanking Cys_{142} (and perhaps Cys_{128}) also contribute to the formation of the cholinergic site. The importance of segment 134–153 may be underestimated in this study because the synthetic peptide is not N-glycosylated. These conclusions are in general agreement with the suggestion of Atassi and co-workers, that several discontinuous segments of the AChR α-subunit sequence could bind ^{125}I-α-BTX, including the segment containing Cys_{128} and Cys_{142} (McCormick & Atassi, 1984; Mulac-Jericevic et al., 1988). Our results are consistent with earlier suggestions, based on the structure of α-neurotoxins (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., (1988) and the properties of their binding to AChR (Martin et al., 1983), that these ligands recognize a large area on the extracellular AChR surface.

The low binding affinity of α-BTX for denatured α subunit or for proteolytic or synthetic α-subunit segments [several orders of magnitude below its affinity for the native AChR (Haggerty & Frohener, 1981; Wilson et al., 1984; Neumann et al., 1986a,b; Gershoni, 1987)], already suggests that the high-affinity binding of α-neurotoxins requires an appropriate tertiary folding of the α chain, including the possibility that several sequence regions are involved in the formation of the cholinergic site. This model is further supported by the results of studies of the binding of α-BTX and small cholinergic ligands to "renatured" or newly synthesized forms of the α subunit. Denatured forms of the α subunit can bind α-BTX, but not, or to a much lesser extent, small cholinergic ligands (Haggerty & Froehner, 1981; Tzartos & Changeux, 1983; Neumann et al., 1986b). Similarly, newly synthesized α subunit acquires the ability to bind α-BTX with low affinity at a much earlier stage of structural maturation than the ability to bind small cholinergic ligands (Merlie, 1984; Carlin et al., 1986). Only after assembly of the α subunit into a mature $\alpha_2\beta\gamma\delta$ AChR molecule is the ability to bind α-BTX with high affinity and curare acquired (Merlie, 1984; Carlin et al., 1986).

α-BTX may recognize partially denatured α subunit, and segments of the α subunit sequence, because it binds to these subsites with sufficiently high affinity. α-Neurotoxins have a similar shape, consisting of three loops arranged side by side (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988). Chemical modifications (Martin et al., 1983) and spectroscopic studies and detailed structural information obtained from X-ray and NMR studies (Walkinshaw et al., 1980; Kistler & Stroud, 1982; Basus et al., 1988) suggest that a large area of the α-neurotoxin molecules formed by three sequence segments, i.e., both sides of the central loop and the lower tip of loop III, is involved in AChR binding. A corresponding large area on the AChR surface, formed by several sequence segments, should make contact with the α-neurotoxins. The high affinity [$K_D = 10^{-12}$–10^{-10} M reviewed in Lee (1979)] and very slow reversibility [$K_{off} \sim 2 \times 10^{-4}$–$5 \times 10^{-5}$ and $4 \times 10^{-3} \times 10^{-4}$ min^{-1} for α-BTX and α-CTX, respectively; e.g., see Blanchard et al., (1979) and Kang and Maelicke (1980)] of the binding of these toxins is consistent with interaction with the AChR via large surfaces, with formation of a large number of attachment points.

The protein nature of α-BTX, the fact that its natural target—the AChR—is a large protein, the size of its binding area, and the high affinity, slow reversibility of its binding with the AChR are all characteristics that α-BTX shares with high-affinity antibodies directed against proteins antigens. It is therefore reasonable to assume that antibodies against the α-BTX binding site will recognize this surface structure in a manner reminiscent of α-BTX binding. Antibodies bind to large areas (690–750 Å), and 14–16 residues on the protein antigen are directly involved in antibody binding [reviewed in Davies et al. (1988)]. Although in all cases studied so far antibody epitopes are formed by juxtaposition of several discrete segments of the antigen sequence, brought in contact as a result of the tertiary folding of the protein, long segments may occur, as in the case of a lysozyme epitope which is formed by 16 residues arranged in two 9-residue loops. A similar situation may occur for the AChR area to which α-BTX and the three mAbs studied here bind, where several residues within the sequence segments α55–74 and α181–200 can still make adequate contact with the mAb or α-BTX and result in efficient binding.

Binding of denatured α subunit or peptides to α-BTX may be improved by "renaturation" in the presence of this high-affinity "matrix". In the same vein, it has been shown that peptides excised from the α-neurotoxin molecule bind with micromolar affinity to native AChR (Guillerot et al., 1981; Martin et al., 1983). Similarly, our high-affinity mAbs, raised against native AChR, could drive toward renaturation small peptides containing constituent segments of their binding site. The α-neurotoxins themselves may undergo structural changes upon binding to the AChR, as suggested by spectral changes of fluorescent toxin upon binding to AChR (Kang & Maelicke, 1980; Johnson et al., 1984; Cheung et al., 1984) and by structural differences of the putative binding surface of crystallized α-CTX and α-BTX, and of crystallized and solubilized α-BTX. In crystals or solutions of α-CTX and in solutions of α-BTX, the proposed area of contact with the AChR is a regular β sheet (Walkinshaw et al., 1982; Sanger et al., 1983; Basus et al., 1988), while in crystals of α-BTX the regularity of the β-sheet configuration of this region is disrupted by an irregular orientation of residue Lys_{27} (Love & Stroud, 1986). It has been proposed that these toxins change their conformation when binding to the AChR (Kistler et al., 1987) and that the structure of crystallized α-BTX may be the true structure of snake α-neurotoxins, when bound to the AChR. In the absence of crystals of the AChR and of complexes between AChR and α- neurotoxins, elucidation of the structure of the complex between α-CTX or α-BTX and peptides Tα181–200 and Tα55–74 may give a preliminary answer to this question.

The binding properties and pattern of mutual inhibition of the mAbs used in the present study suggest that they bind to distinct, overlapping parts of the area recognized by cholinergic ligands and that within this area subsites may esist, recognized either by all small cholinergic ligands or by cholinergic antagonists alone (Watters & Maelicke, 1983; Fels et al., 1986).

Table III[a]

	SEGMENT α55-74
Torpedo californica	RLRQQWIDVRLRWNPADYGG
Mouse	RLKQQWVDYNLKWNPDDYGG
Calf	RLKQQWVDYNLKWNPDDYGG
Human	RLKQQWVDYNLKWNPDDYGG

	SEGMENT α181-200
Torpedo californica	YRGWKHWVYYTCCPDTPYLD
Xenopus laevis	YRCWKHWVYYTCCPDKPYLD
Chicken	YRGWKHWVYYACCPDTPYLD
Mouse	ARGWKHWVFYSCCPTTPYLD
Calf	SRGWKHWVFYACCPSTPYLD
Human	SRGWKHWVTYSCCPDTPYLD

[a] The sequences are from Noda et al. (1983a,b), Boulter et al. (1985), Isenberg et al. (1986), and Merlie et al. (1986).

Indeed, binding subsites for different cholinergic ligands have been suggested long ago, from the results of pharmacological studies (Beers & Reich, 1970).

Although peptide Tα181–200 forms the preferential subsite for the binding of both α-BTX and mAB WF6, different amino acid residues within this sequence segment may be involved in the binding of the two probes. This is suggested by the finding that α-BTX binds preferentially to the oxidized form of Tα181–200, while WF6 prefers the reduced/alkylated form (Figure 8). This may also explain the incomplete inhibition of α-BTX binding by WF6 (Figure 2).

WF6 seems to recognize sequence segments flanking the Cys residues at positions 128 and 142 (Figures 5 and 6). This sequence segment has been previously suggested as part of a cholinergic site because, in addition to the findings summarized in the introduction, a peptide corresponding to α125–147, where the two Cys residues were oxidized to form a disulfide bridge, could bind both α-neurotoxin and acetylcholine (McCormick & Atassi, 1984). Another study, however, did not confirm these results (Criado et al., 1986). Our data favor the possibility that this sequence region is indeed part of, or very close to, the α-BTX binding site. This region contains the only N-glycosylation site in the α subunit (Asn_{141}), which is conserved in all known AChRs from peripheral tissues [reviewed in Maelicke (1988)], and concanavalin A reduces the total α-BTX binding, or at least its association rate, to both *Torpedo* (Wonnacot et al., 1980; Conti-Tronconi et al., 1990) and mammalian muscle (Boulter & Patrick, 1979) AChRs, suggesting that an N-linked sugar moiety is close to the α-BTX binding site. If a carbohydrate domain contributes to α-BTX binding site formation, synthetic peptides, which are not glycosylated, cannot match the structure of the area formed by the sequence region flanking Asn_{141}, and their inability to bind α-BTX could be due to the limitations of the approach.

Because α-BTX binds to all known AChRs from muscle and electric organ, and WF6 cross-reacts among species (Fels et al., 1986), conserved residues should be involved in recognition of these two probes. Table III shows an alignment of the sequence segments α55–74 and α181–200 of the α subunits from *Torpedo* and from different muscle AChRs. Conserved residues and conservative substitutions are indicated by boldface characters. Both segments are highly conserved. Along the segment α55–74 only positions 63, 64, and 70 have nonconservative substitutions. Along the segment α181–200, in addition to Cys_{192} and Cys_{193}, positions 182, 184–188, 190, 194, and 197–200 have identical residues or conservative substitutions in all these AChRs. Binding experiments carried out with synthetic or biosynthetic peptide analogues containing single-residue substitutions will identify which residues are crucial for antibody and α-BTX binding.

Curiously, an important constituent loop of the immunodominant region of the AChR [main immunogenic region, MIR; see Tzartos and Lindstrom (1980)] is located within residues 68–76 (Tzartos et al., 1988; Bellone et al., 1989), and it partially overlaps the segment α55–74. Peptide $T\alpha(67–76)_2$ should contain residues involved in the interaction with α-BTX, because, although it does not bind ^{125}I-α-BTX in dot blot assays, it can inhibit ^{125}I-α-BTX binding to native AChR (Figures 3 and Table II), although less efficiently than Tα55–74. This ability to compete for α-BTX but not to bind it directly in dot blots can be explained if peptide $T\alpha(67–76)_2$ contains only few residues involved in α-BTX binding, so that it binds to α-BTX with low affinity and reversibly. Under such circumstances, its binding to α-BTX could have an effect in the competition experiments, where the peptide concentration is high (approximately 200 μM) and the peptide remains in the incubation mixture during the incubation with ^{125}I-α-BTX. On the other hand, in the dot blot experiments, the amount of peptide present may be insufficient for efficient binding, and/or the quickly reversible nature of the complexes formed would cause the loss of bound ^{125}I-α-BTX during the washings. Anti-MIR antibodies do not interfere with α-BTX binding (Tzartos & Lindstrom, 1980; see also below). Because residues from other sequence regions contribute to form the MIR (Tzartos et al., 1988) and the α-BTX site, the dilemma may be reconciled by assuming that the MIR and the α-BTX site are part of the same surface domain but they overlap only partially, and recognition of the nonoverlapping areas is enough to ensure binding. In addition, the "classic" anti-MIR mAbs were obtained by using a screening procedure which utilized ^{125}I-α-BTX/AChR complexes (Tzartos & Lindstrom, 1980), and only anti-MIR mAbs able to bind in the presence of α-BGT may have been selected. In support of this contention, anti-MIR antibodies are heterogeneous in their binding characteristics, suggesting that they bind to different overlapping epitopes within the MIR (Tzartos & Lindstrom, 1981; Conti-Tronconi et al., 1981). Studies on the binding of anti-MIR mAbs to synthetic peptides corresponding to the MIR region directly demonstrated the existence of several overlapping epitopes within the MIR (Tzartos et al., 1990; Bellone and Conti-Tronconi, unpublished results), and anti-MIR antibodies have been developed which can interfere with α-BTX binding (D. Richman, personal communication).

Because as many as four binding sites for neurotoxins and for cholinergic agonists may exist on the AChR molecule (Maelicke et al., 1977; Dunn et al., 1983; Smart et al., 1984; Conti-Tronconi & Raftery, 1986), the binding of ^{125}I-α-BTX and WF6 to the sequence region of all *Torpedo* AChR subunit homologous to α166–200 was investigated. Only the sequence segment flanking Cys_{192} and Cys_{193} of the α subunit bound, strongly and consistently, both probes. No binding to the other peptides was detected. Therefore, either the α subunit only contains binding sites for α-BTX and/or WF6 or binding sites on subunits other than the α are primarily formed by other sequence segments (e.g., homologous to α55–74), or their binding can occur only to the native AChR molecule, and it is lost for denatured sequences like our synthetic peptides.

In conclusion, this study indicates that the three β strands which form the binding surface of neurotoxins bind to an area of the AChR molecule formed by at least two, and perhaps three, sequence segments and that within this large area subsites for cholinergic agonists or antagonists may exist. The segment flanking Cys_{192} and Cys_{193} and the segment 55–74

of the α subunit contain the major constituent elements of the α-BTX and agonist site, but it is possible that other residues, close to Cys_{142} and perhaps Cys_{128}, contribute to the binding site on the native AChR. Because the mapping of surface structure with short synthetic peptide requires the serendipitous occurrence of high-affinity subsites formed by residues contained in short sequence segments, we cannot exclude or identify other sequence regions which may also contribute residues to the cholinergic site. Our results are in excellent agreement with a recent study (Dennis et al., 1988) which found that a photoaffinity ligand for the acetylcholine binding site labeled in *Torpedo* AChR in an agonist-protectable manner residues Tyr_{149}, Tyr_{190}, and $Cys_{192,193}$ and other nonidentified residue(s) in the segment α31–105. A binding site formed by several sequence regions, stabilized by disulfide bridges and including a glycosylated moiety, would be both capable of sensitive differentiation between different ligands and amenable to various means of modulation of the recognition function (e.g., by changes in glycosylation and/or disulfide bridge formation).

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Voltage Activation of Purified Eel Sodium Channels Reconstituted into Artificial Liposomes†

Ana M. Correa,*,‡ Francisco Bezanilla,‡ and William S. Agnew§

Department of Physiology, Ahmanson Laboratory of Neurobiology and Jerry Lewis Neuromuscular Research Center, University of California School of Medicine, 405 Hilgard Avenue, Los Angeles, California 90024, and Department of Cellular and Molecular Physiology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

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ABSTRACT: We report here a characterization of the voltage-activated behavior of sodium channels purified from the electroplax of *Electrophorus electricus*. Single-channel activity in response to depolarizing pulses was recorded from patches excised from liposomes containing the reconstituted channel. Strong hyperpolarizations were required to elicit channel activity. Channels exhibited two typical gating patterns. They either would open in brief bursts upon depolarization and then inactivate (fast) or would stay opened for prolonged periods that frequently lasted several consecutive depolarizations and showed intense flickering (slow). The single-channel conductance estimated from the slope of the *I-V* curves ranged between 15 and 30 pS under several experimental conditions. Channels gating in either mode, fast or slow, were indistinguishable in terms of their sizes. No clear difference in their mean open times was observed. In addition to the two gating patterns, we also found a very clear tendency of the channels to stay quiet for long periods.

In the past several years, voltage-sensitive sodium channels have been isolated biochemically from a variety of electrically excitable tissues, including eel electroplax (Agnew et al., 1978; Miller et al., 1983; Norman et al., 1983; James et al., 1989), rat and rabbit skeletal muscle (Barchi et al., 1980; Barchi, 1983; Kraner et al., 1985), chicken heart (Lombet & Lazdunski, 1984), and rat brain (Hartshorne & Catterall, 1981, 1984; Barhanin et al., 1983). One question which has remained largely unanswered was whether these purified proteins could be reassembled into artificial membranes and induced to function normally in response to changes in the transmembrane potential. Single-channel recording by patch-clamp techniques offers extremely sensitive measurements of channel gating and conductance. The present report describes progress in applying patch-clamp recording techniques to the characterization of pure sodium channel molecules which have been isolated from eel electroplax and reconstituted into artificial liposomes. This approach is demonstrated to be suitable for detailed characterization of the conductance and gating properties of these molecules under well-defined conditions of membrane protein and lipid composition.

Sodium channels from all tissues so far examined contain a large heavily glycosylated peptide, or α-subunit, of

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* Author to whom correspondence should be addressed.
‡ University of California School of Medicine.
§ Yale University School of Medicine.

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