Evidence That the Acetylcholine Binding Site Is Not Formed by the Sequence α127–143 of the Acetylcholine Receptor†

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ABSTRACT: The sequence α127–143 of the α subunit of the acetylcholine receptor has been proposed to contain several important features: (1) the acetylcholine binding site, (2) the only N-glycosylation site of the α subunit, at asparagine-α141, and (3) two cysteine residues, at α128 and α142, that may participate in a disulfide bond known to be near the binding site. We tested these hypotheses by using antisera to receptor and its subunits and monoclonal antibodies to the synthetic peptide α127–143 cyclized by a disulfide bond between α128 and α142. Antisera to receptor and its α subunit were able to immunoprecipitate the iodinated peptide, and this reaction was inhibited by soluble receptor, but not by membrane-bound receptor. α-Bungarotoxin did not inhibit antiserum binding to solubilized receptor. Similarly, cholinergic ligands had little or no effect on binding to immobilized receptors of anti-peptide monoclonal antibodies. In addition, these monoclonal antibodies, when bound to the receptor, did not affect toxin binding kinetics. By contrast, preincubation with concanavalin A did inhibit monoclonal antibody binding. Reduction of the receptor significantly decreased the binding of three of the monoclonal antibodies, but subsequent alkylation with *N*-ethylmaleimide or the affinity labeling reagent bromoacetylcholine had no additional effect on binding. A dithiothreitol concentration about 100-fold higher than the one needed to reduce the disulfide near the acetylcholine binding site was necessary to inhibit monoclonal antibody binding. We conclude that the sequence α127–143 (1) is not fully exposed on the surface when the receptor is in the membrane, (2) is probably glycosylated at asparagine-α141, (3) may have cysteines-α128 and -α142 forming a disulfide bond which is not related to the disulfide known to be close to the cholinergic binding site, and (4) is not involved in the formation of the binding site for cholinergic agonists or antagonists.

The nicotinic acetylcholine receptor is involved in neurotransmission at synapses between motor nerves and vertebrate skeletal muscles or fish electric organs [for recent reviews, see Cold Spring Harbor Laboratory (1983)]. The receptor is composed of four homologous subunits with the stoichiometry $\alpha_2\beta\gamma\delta$. Binding of acetylcholine to its α subunits triggers opening of a cation channel through the receptor.

With the availability of the sequences of cDNAs for receptor subunits (Noda et al., 1983; Claudio et al., 1983; Devillers-Thiery et al., 1983), structural and functional features are being assigned to amino acid sequences of receptor subunits. In vitro mutagenesis has been used to provide evidence that α subunits are glycosylated at asparagine-α141 (Mishina et al., 1985). Affinity labeling has been used to provide evidence that cysteine-α192 participates in a disulfide bond near the acetylcholine binding site (Kao et al., 1984), and sequence-specific antibodies have been used to extensively test, and revise, theoretical models for the transmembrane orientation of the subunit polypeptide chains (Lindstrom et al., 1984; Ratnam & Lindstrom, 1984; Criado et al., 1985a,b; Young et al., 1985; La Rochelle et al., 1985; Ratnam et al., 1985a,b).

Noda et al. (1982) initially proposed that cysteines-α128 and -α142 were linked by a disulfide bond and that the resulting loop formed the acetylcholine binding site. They proposed this because (1) it was a likely site for glycosylation, which might locate this sequence on the extracellular surface, (2) it was known from affinity labeling studies (Karlin, 1969) that a disulfide bond was located near the acetylcholine binding site, (3) secondary structure predictions suggested a possible pleated-sheet structure reminiscent of other binding sites, and (4) amino acid residues were present which could be imagined to interact with acetylcholine. The idea that this sequence might form the acetylcholine binding site has become quite popular (Smart et al., 1984; McCormick & Atassi, 1984; Boulter et al., 1985; White, 1985). However, there is no experimental evidence that this sequence forms the acetylcholine binding site. In fact, observation that an acetylcholine binding site affinity label reacts with cysteines-α192 and -α193 (Kao et al., 1984) could argue that the acetylcholine binding site was formed by a sequence near α192. An intermediate proposition, also without experimental support, is that in α subunits there are double disulfide bonds linking cysteines at α128, 142, 192, and 193 (Kao et al., 1984; Boulter et al., 1985). A possible argument against the acetylcholine binding site being formed by a disulfide-linked loop between α128 and α142 is that cysteines in these positions are conserved in β, γ, and δ subunits and that much of the amino acid sequence between them is also conserved (Noda et al., 1983), yet it is clear from affinity labeling studies (Karlin, 1980) and toxin binding studies (Wilson et al., 1984) that the acetylcholine binding sites which regulate opening of the cation channel are unique to α subunits.

In this paper, we report experimental tests of the proposition that amino acids contained in α128–142 form the acetylcholine binding site. We found that antibodies to the corresponding synthetic peptide do not compete for binding to receptors with cholinergic ligands. Therefore, we conclude that this sequence

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does not form the acetylcholine binding site. Further, we obtained evidence that this sequence is not clearly exposed on the extracellular surface of receptors in the membrane and evidence which suggests that cysteines-α128 and -α142 are not involved in disulfide bonds adjacent to the acetylcholine binding site. Our data are consistent with the idea that α subunits are glycosylated at α141.

Materials and Methods

Synthetic Peptide α127–143. The synthetic peptide YCEIIVTHFPFDQQNCT corresponding to the sequence of *Torpedo californica* α-subunit sequence 127–143 (Noda et al., 1982) was synthesized on an Applied Biosciences synthesizer and disulfide linked between the cysteines at positions 128 and 142 (by V. Sarin). The fully protected peptide resin was synthesized from *tert*-butyloxycarbonyl (Boc)[1]-threonine–[[4-(oxymethyl)phenyl]acetamido]methyl resin (Boc-Thr–OCH_2Pam) by normal stepwise solid-phase peptide synthesis using the preformed symmetric anhydride of all protected amino acids except for Boc-glutamine and Boc-asparagine where a DCC/HOBT coupling protocol was used. The free peptide was liberated from the synthesized peptide resin by using anhydrous HF and then extracted into 15–25% aqueous acetic acid. After lyophilization, the peptide was oxidized to form a disulfide bond by using 0.01 M $K_3Fe(CN)_6$. The peptide was dissolved at a final concentration of 1×10^{-5} M in 0.1 M ammonium acetate buffer at pH 7.41. The completeness of the reaction was monitored by Ellman's reaction. The oxidized peptide was purified by gel filtration on Sephadex G-15 (where it gave a sharp peak in the included volume) followed by reverse-phase (Vydac C_4) HPLC.

The peptide was labeled with ^{125}I to a specific activity of 2×10^{18} cpm/mol by using chloramine T but no reducing step according to the same basic method we use for labeling α-bungarotoxin except that Bio-Gel P-2 was substituted for Sephadex G-25 in the column used to separate labeled peptide from free ^{125}I (Lindstrom et al., 1981).

Antibodies to Receptor. Antisera to affinity-purified receptor and its component subunits were prepared as previously described (Lindstrom et al., 1979b). Monoclonal antibodies (mAbs) which react with denatured α subunits have also previously been reported (Tzartos & Lindstrom, 1980; Tzartos et al., 1981, 1985; Gullick & Lindstrom, 1983). Reaction of antibodies with ^{125}I-labeled synthetic peptide used triplicate 100-μL aliquots of 5 nM peptide in 0.5% Triton X-100, 100 mM NaCl, 10 mM sodium phosphate buffer pH 7.5, and 10 mM NaN_3. Antisera (5 μL) or mAbs (5 μL of stocks of 1–100 μM plus 3 μL of normal rat serum carrier) were added overnight at 4 °C; then 100 μL of a dilution of goat anti-rat IgG was added for 30 min at 4 °C. After dilution to 1 mL, pelleting in a microfuge, and two subsequent 1-mL washes, ^{125}I in the pellets was quantitated by γ counting. Normal serum blanks were used and these values subtracted from experimental values. Competition experiments including receptor in various forms during the overnight incubation, and when membrane-bound receptor was used, the detergent Triton X-100 was omitted from the buffer.

Anti-Peptide Monoclonal Antibodies. The synthetic peptide α127–143 was coupled to keyhole limpet hemocyanin by reaction with bis(diazobenzidine) (Bassiri & Utiger, 1979). The peptide (3 mg) was dissolved in 0.8 mL of 130 mM NaCl/160 mM sodium borate buffer, pH 9.0, and 4 mg of keyhole limpet hemocyanin (Calbiochem-Behring) was added. Then 160 μL of 9 mM bis(diazobenzidine) was added at 0 °C with vigorous shaking and the pH adjusted to 9 by adding 62.5 μL of 0.5 N NaOH. After 2 h at 4 °C in the absence of light, aliquots were diluted with 100 mM NaCl/10 mM sodium phosphate, pH 7.4, and frozen. Female Lewis rats were injected intradermally in multiple sites with 100 μg of coupled peptide emulsified with complete Freund's adjuvant in a 200-μL volume on days 0, 14, and 36 and injected intraperitoneally with 100 μg of the conjugated peptide on days 56 and 57. On day 60, hybridoma cells were obtained by fusing the mouse myeloma cell line S194 with spleen cells of the immunized rats, as previously described (Tzartos & Lindstrom, 1980; Hochschwender et al., 1985). Hybridoma lines secreting antibodies were selected by ELISA using purified α subunits bound to microwells. Five hybridoma cell lines secreting the mAbs 257, 258, 259, 260, and 261 were obtained.

Solid-Phase Enzyme-Linked Immunosorbent Assay (ELISA) and Radioimmunoassays. For screening the clones and investigation of mAb specificity, either ELISA or radioimmunoassays were used (Hochschwender et al., 1985). Briefly, purified subunits (Lindstrom et al., 1979b), receptor (Lindstrom et al., 1980), or receptor-rich membranes (Lindstrom et al., 1980) were bound to microtiter dishes (Immulon I, Dynatech) by adding 50 μL/well of a 2×10^{-8} M solution in 10 mM sodium bicarbonate buffer, pH 9.5, overnight at 4 °C. This buffer treatment does not reduce the ability of receptor to bind ^{125}I-α-bungarotoxin. Unreacted sites were quenched by addition of 0.5% bovine serum albumin in 0.5% Tween 20, 100 mM NaCl, and 10 mM sodium phosphate, pH 7.4, for 15 min, except in radioimmunoassays or assays with membrane-bound receptor, in which cases Tween was omitted. Quenching was followed by four washes (200 μL/well) with the same solution. mAbs were allowed to bind for 4 h at room temperature. After another three washes, mAb binding was detected by using a mAb to rat IgG κ chains [MAR 18.5; see Lanier et al. (1982)] coupled to glucose oxidase (Harper & Orengo, 1981) and mixed with goat anti-rat IgG coupled to the same enzyme. In the case of radioimmunoassays, a saturating concentration of ^{125}I-labeled affinity-purified goat anti-rat IgG (10 nM) was used to detect mAb binding. After 2.5 h at room temperature, wells were washed 5 times and counted. With the exception of the preliminary screenings and experiments to determine subunit specificity, in which ELISA was used, all other experiments involved the use of radioimmunoassay in solid phase as described above. This permitted a more precise quantitation of mAb binding, although in most cases a good correlation with ELISA results has been found.

Protein blotting from electrophoretic gels was done on Zeta-probe blotting membranes (Bio-Rad). Probing of the blots with various antibodies was done essentially as previously described (Gullick & Lindstrom, 1983) by incubating first with the corresponding mAb (100 nM) overnight at room temperature, washing, and then incubating with ^{125}I-labeled affinity-purified goat anti-rat IgG for 4 h at room temperature. After overnight preincubation with mAbs (100 nM), the binding of ^{125}I-α-bungarotoxin (10 nM) was performed for 5 h, always in the presence of the same concentration of mAb.

Results and Discussion

Antibodies to the sequence α127–143 cyclized by a disulfide

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; HOBT, 1-hydroxybenzotriazole hydrate; IgG, immunoglobulin G; mAb, monoclonal antibody; HPLC, high-pressure liquid chromatography; BSA, bovine serum albumin; Con A, concanavalin A; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; KLH, keyhole limpet hemocyanin; BrAcCh, bromoacetylcholine; Carb, carbamylcholine.

Table I: Detection of Antibodies to ^{125}I-α127–143 by Immunoprecipitation[a]

antibody	cpm − control cpm
antisera to	
intact receptor	183000
α subunits	205000
β subunits	−86
γ subunits	−158
δ subunits	−164
17 mAbs to main immunogenic region on α	−227 (av)
17 mAbs to other sites on α	−218 (av)

[a] ^{125}I-α127–143 (5 nM) was incubated overnight in triplicate 100-μL aliquots with 5 μL of serum or 5 μL of mAb plus 3 μL of normal serum. Then anti-antibody was added. ^{125}I in the washed immune precipitates was determined, and a control value for 5 μL of normal rat serum was subtracted.

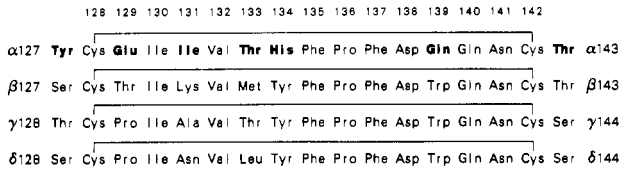

FIGURE 1: Comparison of α127–143 with homologous sequences of β, γ, and δ subunits. Sequence data are from Noda et al. (1982). The disulfide bond shown exists in the synthetic peptide we used and has been proposed to exist in receptor (Noda et al., 1982), but it has also been proposed that in α subunits cysteines-α128 and -α142 are involved in disulfide bonds with cysteines at positions α192 and α193 which are unique to α subunits (Kao et al., 1984; Boulter et al., 1985).

bond between cysteines at positions 128 and 142 were sought first by screening existing antisera and monoclonal antibodies (mAbs) for reaction with this ^{125}I-labeled peptide (Table I). Antisera to both native receptor and purified denatured α subunits reacted at greater than 40-fold background. Comparing the moles of receptor monomers bound per liter by antisera to α (1×10^{-6} M) with the moles of peptide bound per liter (2×10^{-8} M) suggests that on the order of 1.0% of these antibodies to α recognize the sequence α127–143. None of 34 mAbs specific for α subunits bound to this sequence. Neither was this sequence recognized by antisera to β, γ, or δ subunits. This is interesting because there is substantial sequence homology between α, β, γ, and δ sequences in this region (Figure 1). It is generally supposed that six to eight amino acids are involved in forming an antibody binding site (Atassi, 1984), although the area of a protein occluded by a bound antibody is probably substantially larger. In the sequence α127–134, five of eight amino acids differ between α subunits and all three of the other subunits. Therefore, it is likely that the serum antibodies which bind to α127–143 bind to region α127–134.

Five mAbs were obtained against the synthetic cyclic peptide α127–143. Four of them, mAbs 257, 258, 259, and 260, like the antisera, were specific for the α subunit of the receptor, while the remaining one, mAb 261, cross-reacted with the other subunits (Table II). The affinity of these mAbs for receptor bound to microwells was quite high (Table III); however, these mAbs were ineffective at binding to soluble receptor or α subunits in immune precipitation assays. It may be that binding of receptor to the plastic alters its conformation somewhat. In any case, α-bungarotoxin and other ligands are able to bind to immobilized receptor, suggesting that the acetylcholine binding site is still in good shape. mAbs 258 and 259 had high affinity for both the synthetic peptide and the intact receptor, whereas mAb 260 had higher affinity for the peptide than for the intact receptor (Table III). mAb 261, which cross-reacted with other subunits, exhibited similar

Table II: Binding of mAbs against the Synthetic Peptide α127–143 to Receptor Subunits Measured by ELISA[a]

	absorbance at 410 nm ± SD (% of binding to α subunit)			
mAb	α	β	γ	δ
257	1.2 ± 0.1 (100)	0.0 (0.0	0.0 (0.0)	0.0 (0.0)
258	1.2 ± 0.0 (100)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
259	1.3 ± 0.0 (100)	0.1 ± 0.0 (7.7)	0.0 (0.0)	0.0 (0.0)
260	0.8 ± 0.1 (100)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
261	1.0 ± 0.1 (100)	1.0 ± 0.0 (100)	0.5 ± 0.0 (50)	0.1 (10)

[a] Purified subunits were immobilized on microwells (1 pmol/well). mAbs (3–6 nM) were allowed to bind for 4 h. After the wells were washed, bound mAbs were quantitated by using anti-antibody conjugated to glucose oxidase.

Table III: Affinities of mAbs to α127–143 for Intact Receptor and α127–143 Coupled to KLH[a]

	K_D (nM)	
mAb	receptor	α127–143
257	3.1	390
258	0.28	2.9
259	0.70	13
260	97	1.1
261	23	1000

[a] Purified receptor or peptide α127–143 coupled to KLH was immobilized on microwells, and mAbs were added at different concentrations and allowed to bind for 4 h. The amount of mAb bound was detected in each case by ^{125}I-labeled goat anti-rat IgG. A calibration curve was made with affinity-purified rat IgG and ^{125}I-labeled goat anti-rat IgG to permit calculation of moles of mAb bound.

Table IV: Binding of mAbs to Receptors in Membranes[a]

	% of binding to purified receptor	
mAb	native vesicles	pH 11.2 treated vesicles
257	33	32
258	5.5	12
259	7.6	14
260	22	27
261	47	36

[a] Purified receptor, native (Lindstrom et al., 1980) or pH 11.2 treated (Neubig et al., 1979; Ratnam et al., 1985a,b) receptor-rich membranes at the same concentration of α-bungarotoxin binding sites (1 pmol/well), was immobilized on microwells. Then mAb (3.5 nM) binding proceeded for 4 h. The amount of mAb bound was detected by ^{125}I-labeled goat anti-rat IgG. To normalize experiments using solubilized and membrane-bound receptors, the amount of ^{125}I-α-bungarotoxin binding sites present in each well (0.08–0.12 pmol/well) was determined in parallel experiments. Then the number of mAbs bound per toxin binding site was calculated for receptor. These values are compared in the table.

moderate affinity for receptor but low affinity for the peptide. Perhaps the conformation of the sequence in intact receptor subunits conferred higher affinity for mAb 261.

The acetylcholine binding site is exposed on the extracellular surface of the receptor to permit interaction with acetylcholine released from the nerve. Therefore, we investigated the location of α127–143 in the receptor. Addition of receptor-rich membrane vesicles sealed righ-side-out to the radioimmunoassay did not inhibit the binding of serum antibodies to the labeled peptide (Figure 2). This indicates that the part of this sequence which is unique to α subunits is not readily accessible on the surface of membrane-bound receptors. mAbs 258 and 259, the mAbs with highest affinity for detergent-solubilized receptor bound to microwells (Table III), bound poorly to receptor-rich membrane vesicles bound to microwells (Table IV). Extraction of the vesicles at pH 11.2 removes extrinsic proteins from the cytoplasmic surface (Neubig et al., 1979) and permeabilizes them (Ratnam et al., 1986a,b), but only slightly increased antibody binding (Table IV). mAb 261,

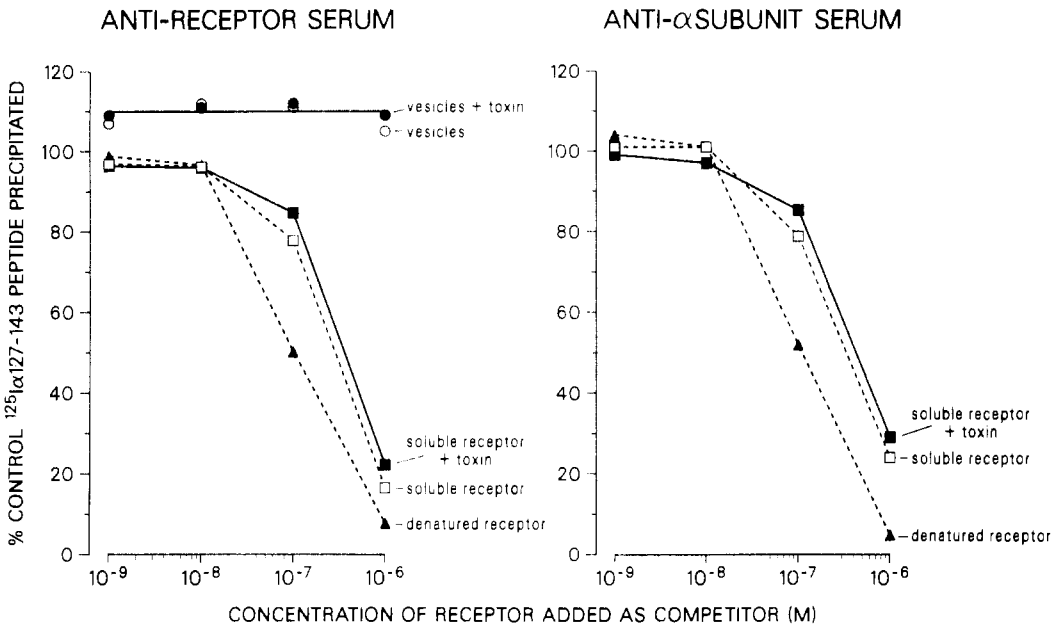

FIGURE 2: Inhibition of binding of anti-receptor or anti-α-subunit sera to the peptide ^{125}I-α127–143 by receptor in various forms. Antiserum (1×10^{-9} M anti-peptide) and ^{125}I-α127–143 peptide (5×10^{-9} M) were incubated overnight alone or in the presence of receptor at the indicated concentrations. (○) Receptor-rich membrane vesicles; (●) vesicles plus α-bungarotoxin (5×10^{-6} M); (□) affinity-purified Triton-solubilized receptor; (■) soluble receptor plus α-bungarotoxin (5×10^{-6} M); (▲) soluble receptor denatured with 1% SDS.

a lower affinity mAb, which cross-reacted with other subunits and, therefore, bound to a conserved sequence not recognized by mAbs 258 and 259, bound to membrane-bound receptors more effectively than did mAbs 258 and 259. This suggests that the part of the sequence α127–143 which is conserved between subunits (α135–143) is more exposed on the surface of membrane-bound receptors than is the part which is unique to α subunits (α127–134). If α127–143 formed the acetylcholine binding site, the amino acids unique to α would be expected to contact acetylcholine and exposed on the surface. Since it is known that mAbs to the acetylcholine binding site can bind to receptors in membranes (Sourojon et al., 1983), the observation that the mAbs to α127–134 did not bind well to receptors in membranes suggests that this sequence does not form the acetylcholine binding site.

It is known that mAbs can be made to the acetylcholine binding site and that their binding can be inhibited both by α-bungarotoxin and by small cholinergic ligands (James et al., 1981; Gomez et al., 1981; Walters & Maelicke, 1983; Sourajon et al., 1983; Whiting et al., 1985). Therefore, if α127–143 formed the acetylcholine binding site, mAbs to this sequence should compete for binding to receptor with α-bungarotoxin and small cholinergic ligands. In several experiments, some of which are shown in Figure 2, serum antibodies bound to the same extent to solubilized receptor whether or not its acetylcholine binding sites were bound with α-bungarotoxin. Solubilized receptors bound to microwells were shown to retain their ability to bind α-bungarotoxin. Only about 10% of receptors applied to the wells were bound, and most (80%) of the remaining ^{125}I-α-bungarotoxin binding activity could be recovered in the supernatant. Several cholinergic ligands were tested for their ability to inhibit binding to receptors on microwells of mAbs 257, 258, 259, 260, and 261 (Table V). High concentrations of the agonist carbamylcholine and the antagonist benzoquinonium inhibited binding of the mAbs little, if at all, while they completely inhibited binding of ^{125}I-α-bungarotoxin to immobilized receptor (data not shown). Very high concentrations (10^4-fold molar excesses) of the antagonists *d*-tubocurarine and α-bungarotoxin produced some inhibition, but in no case did it surpass 25%. These antagonist concentrations completely blocked binding of ^{125}I-α-bungarotoxin to receptor. mAbs 258 and 259 in saturating concentrations also did not reduce either the initial rate or the final amount of ^{125}I-α-bungarotoxin binding to receptors on microwells (data not shown). As an alternative approach to testing for competitive binding between mAbs and α-bungarotoxin, protein blots of purified receptor were probed with mAbs and ^{125}I-α-bungarotoxin (Figure 3). mAbs 258 and 259, as expected from ELISA experiments (Table II), bound only to α subunits. ^{125}I-α-Bungarotoxin also bound to α subunits, and addition of excess mAb 258 or 259 did not reduce this binding. Thus, in several experimental approaches, there was no evidence that antibodies to α127–143 competed with cholinergic ligands for binding to receptor. This strongly suggests that this sequence does not form the acetylcholine binding site.

Table V: Effect of Cholinergic Ligands on Binding of mAbs 257–261 to Receptor[a]

	% of binding to purified receptor (or native vesicles)[b] without ligand preincubation			
mAb	Carb (1 mM)	*d*-tubocurarine (1 mM)	benzoquinonium (1 mM)	α-bungarotoxin (10 μM)
257	96 (90)[b]	82 (80)	96 (100)	93 (100)
258	95	83	98	75
259	93	88	95	82
260	100 (110)	73 (85)	82 (89)	83 (77)
261	100 (100)	75 (92)	89 (96)	110 (120)

[a] Purified receptors (or native vesicles) were immobilized on microwells, and before incubation with mAbs (~3.5 nM), they were preincubated with different ligands at the indicated concentrations for 1 h. During incubation with mAbs, the same ligand concentration was maintained. mAb binding was detected as in Table II. [b] The results obtained with native vesicles are indicated in parentheses.

The effects of reduction and nonspecific alkylation or affinity alkylation of the acetylcholine binding site were tested on the ability of mAbs to α127–143 to bind to receptors (Table VI). Binding of mAbs 257 and 261 was not affected by reduction of the receptor with dithiothreitol (DTT) or subsequent alkylation with *N*-ethylmaleimide, whereas binding of mAbs 258, 259, and 260 was decreased (Figure 4). These results suggest

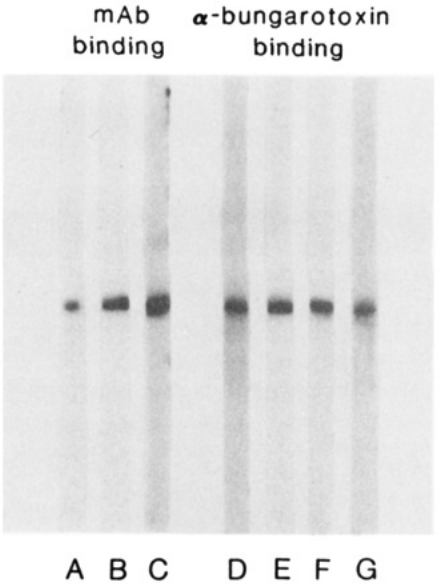

FIGURE 3: Binding of ^{125}I-α-bungarotoxin to the α subunit of the receptor is not affected by mAbs to α127–143. Purified receptor denatured with sodium dodecyl sulfate was electrophoresed on a 10% polyacrylamide gel and transferred electrophoretically to a Zeta-probe blotting membrane. α subunits were located by using mAb 210 to the main immunogenic region followed by ^{125}I-labeled goat anti-rat IgG (lane A). mAbs 258 and 259 to α127–143 were similarly shown to bind to α subunits (lanes B and C, respectively). In lanes D–G, α subunits are labeled with ^{125}I-α-bungarotoxin. Lane D is a buffer control, whereas lanes E–G were preincubated with 100 nM mAb, either mAb 210 (E), mAb 258 (F), or mAb 259 (G), before incubation with toxin.

Table VI: Effect of Reduction with Dithiothreitol and Alkylation with *N*-Ethylmaleimide or Bromoacetylcholine on Binding of mAbs to α127–143 to Receptor[a]

	% of binding to control (without any treatment)		
mAb	DTT (1 mM)	DTT (1 mM) + NEM (10 mM)	DTT (1 mM) + BrAcCh (10 mM)
257	100 (100)[b]	94 (100)[b]	89 (69)[b]
258	69 (56)	75 (57)	85 (55)
259	74 (58)	78 (75)	86 (57)
260	54 (70)	66 (73)	66 (60)
261	100 (110)	98 (88)	85 (88)

[a] Purified receptor or native vesicles were immobilized on microwells and after the wells were washed (200 μL/well) with 100 mM NaCl/10 mM sodium phosphate buffer, pH 7.4, they were reduced with 1 mM dithiothreitol in 150 mM NaCl, 1 mM EDTA, and 3 mM Tris buffer, pH 8.3 (100 μL/well), for 30 min at room temperature. They were subsequently alkylated with 10 mM *N*-ethylmaleimide or bromoacetylcholine in 1 mM dithiothreitol, 50 mM NaCl, 1 mM EDTA, 3 mM NaN_3, and 10 mM sodium phosphate buffer, pH 7.0 (100 μL/well), for 30 min at room temperature. After the wells were washed 3 times, the same procedure as in Tables IV and V was followed for binding of mAbs. Results are expressed as a percentage of a control in the same incubation conditions but without reducing and alkylating agents. [b] In parentheses are indicated the reults obtained with native vesicles under the same reaction conditions.

that these two groups of mAbs bind to slightly different sequences whose conformations may be differentially effected by reduction and alkylation of disulfide bonds involving cysteine-α128 and -α142. Kao et al. (1984) have shown that [4-(*N*-maleimido)benzyl]trimethylammonium, an affinity labeling reagent for the acetylcholine binding sites which labels receptors after reduction of a disulfide bond near the acetylcholine binding site, reacts at cysteine-α192. Bromoacetylcholine is another affinity labeling reagent which similarly requires reduction of the receptor (Damle et al., 1978) and which probably reacts at the same site. We used bromoacetylcholine under conditions (Wolosin et al., 1980) which we found (Figure 5) to cause blockage of >90% of ^{125}I-α-bungarotoxin binding to receptors on microwells. The fact that bromoacetylcholine did not affect mAb binding further confirms that the sequence α127–143 is not part of the acetylcholine binding site.

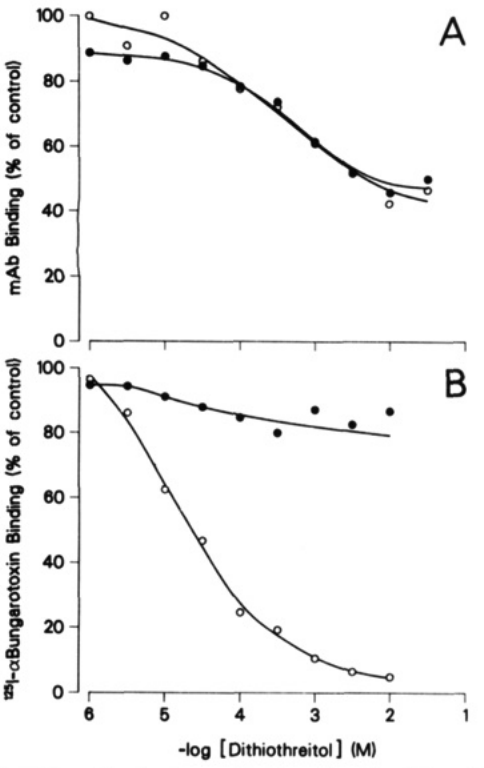

FIGURE 4: Effect of reduction on binding of mAbs 258 and 259 and α-bungarotoxin to receptors. Receptor immobilized on microwells was reduced with different concentrations of DTT for 30 min at room temperature. After the receptor was washed 3 times, binding of mAbs 258 [panel A (○)] and 259 [panel A (●)] was measured by using ^{125}I-labeled goat anti-rat IgG (as in Tables IV and V). The binding of ^{125}I-α-bungarotoxin (panel B) was determined in parallel experiments in which the receptor was reduced with different concentrations of DTT and subsequently alkylated [panel B (○)] or not alkylated [panel B (●)] with 1 mM bromoacetylcholine (as in Table VI).

Our data, which strongly suggest that α127–143 does not form the acetylcholine binding site, are consistent with other data using synthetic peptides. Plumer et al. (1984) raised mAbs to the synthetic peptide α127–132 and, like us, found that their binding to receptor was not inhibited by cholinergic ligands. In their experiments, unlike ours, mAbs were reported to bind equally well to solubilized and membrane-bound receptors in their microwells; however, their ELISA was apparently conducted with 0.05% Tween, a detergent concentration which might be expected to effectively solubilize their receptors. Newmann et al. (1985) raised antisera to α126–143 and observed that these bound to a V8 protease generated fragment of α subunits which did not bind α-bungarotoxin. This is also consistent with our results. Wilson et al. (1985) reported that the synthetic peptide α173–204 bound α-bungarotoxin (IC_5 = 0.1 μM) and that binding was inhibited by curare (IC_5 = 0.1 mM). These results suggest that amino acids contributing to the acetylcholine binding site are in fact located in the sequence α173–204, not α127–142. In contrast to all of these results, McCormick and Atassi (1984) report that the synthetic peptide α125–147 binds ^{125}I-α-bungarotoxin and [^{3}H]acetylcholine. Strangely, to measure ^{125}I-α-bungarotoxin binding, receptor and peptide were coupled to agarose. Only 0.02% as much ^{125}I-α-bungarotoxin binding was

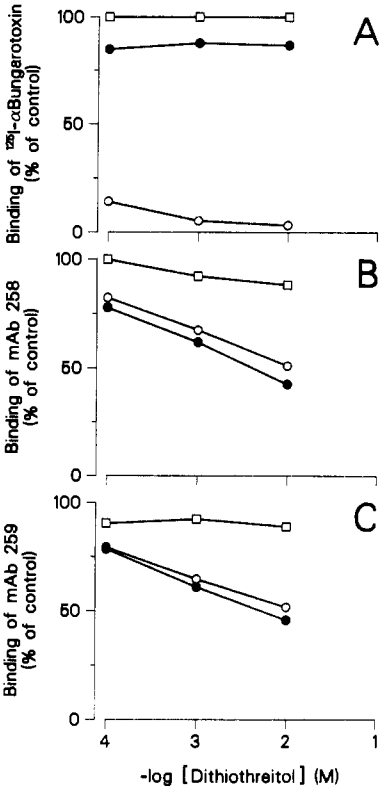

FIGURE 5: Effect of reoxidation on the binding of mAbs 258 and 259 and α-bungarotoxin to reduced receptor affinity labeled with bromoacetylcholine. Receptor immobilized on microwells was reduced with DTT for 30 min and then either left untreated (●), reoxidized with 1 mM DTNB for 30 min in 50 mM Tris, pH 8.0, and 2 mM EDTA (□), or affinity labeled with 1 mM bromoacetylcholine (○). After three washes, binding of ^{125}I-α-bungarotoxin (A), mAb 258 (B), or mAb 259 (C) was measured (as in Tables IV–VI).

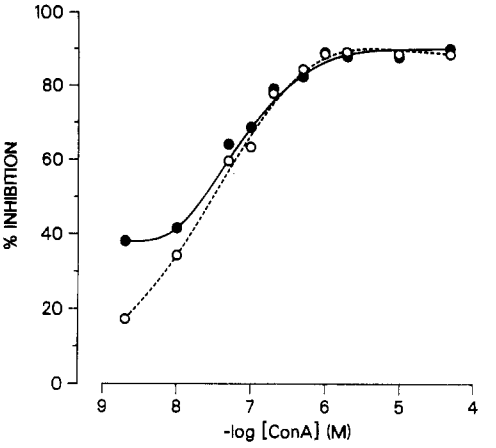

FIGURE 6: Inhibition of binding of mAbs 258 and 259 to receptor by increasing concentrations of concanavalin A. Purified receptor was immobilized on microwells and preincubated with concanavalin A (150 mM NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.5 BSA, and 10 mM sodium phosphate, pH 7.4) for 1 h at room temperature before incubation with mAbs 258 (○) or 259 (●) (~10 nM) was started. Then the binding of the mAbs was quantitated by using ^{125}I-labeled goat anti-rat IgG.

observed to the coupled receptor as would be expected due to the amount of receptor coupled, perhaps due to inactivation during coupling. About 100-fold molar excess of coupled peptide was reported to bind about 37% as much ^{125}I-α-bungarotoxin as did this amount of receptor. No protection experiments were reported. They also reported that a mixture of peptide and [^{3}H]acetylcholine separated on Sephadex G25 showed binding of 0.87 mol of acetylcholine per mole of peptide. This binding required 3 h at room temperature at physiological pH. Acetylcholine is notoriously labile to hydrolysis. Their cyclic peptide would have lysine-α125 across from lysine-α145. It is possible that these might be able to bind [^{3}H]acetate derived from hydrolyzed acetylcholine. After reduction, they observed no binding. They report no competitive binding experiments with cholinergic ligands, choline, or acetate. Whatever the explanation for their observations, their conclusion that they synthesized the acetylcholine binding site is unlikely to be correct.

It has been suggested that cysteines-α128 and -α142 may either be linked by a disulfide bond (Noda et al., 1982; Mishina et al., 1985) or be linked to cysteines-α192 and -193 in double disulfide bonds (Kao et al., 1984; Boulter et al., 1985). We tried to distinguish between these alternatives. In order to monitor the reduction of a disulfide bond involving cysteine-α192, we measured inhibition of ^{125}I-α-bungarotoxin binding by bromoacetylcholine added after reduction. Reduction of disulfide bonds involving cysteines-α128 and -142 was monitored by measuring the inhibition of binding of mAbs 258 and 259 caused by reduction. It could be argued that a general conformational change of the receptor upon its reduction, rather than the precise reduction of cysteines-α128 and -α142, would produce this inhibitory effect, but then mAbs 257 and 261, directed to the same area, or mAb 237 directed against α152–159 should be affected in a similar way, and they were not. As shown in Figure 4, the concentration of DTT needed to reduce 50% of the disulfide close to the binding site was about 25 μM, whereas the concentration of DTT required to produce 50% inhibition of mAb binding was 3 mM, 100-fold more. This result suggests that the disulfide which affects the binding of mAbs 258 and 259 is not the one close to the acetylcholine binding site. An alternative explanation to account for this result is that after reduction and during mAb binding (~4 h) the generated sulfhydryl groups are rapidly reoxidized, unless a very high DTT concentration was used for reduction. During the parallel experiment of inhibition of toxin binding, this high DTT concentration would not be necessary, given that the sulfhydryl groups are immediately alkylated by bromoacetylcholine. However, this possibility was eliminated when it was observed that the inhibition of mAb binding produced by DTT was not modified by bromoacetylcholine (Figure 5, panels B and C). However, reoxidation by 1 mM DTNB after reduction by DTT (Figure 5) restored mAb binding.

Additional evidence that the disulfide close to the binding site is not the same one which affects mAb binding was obtained. After reduction with DTT (10 mM) and affinity alkylation with bromoacetylcholine (10 μM), 95% of the toxin binding sites were blocked, and mAb binding was inhibited by 48%. However, subsequent DTNB reoxidation (1 mM) restored 98% of the binding of mAbs 258 and 259, while the binding of α-bungarotoxin remained 95% blocked. These results clearly show that when the cysteines near the acetylcholine binding site (α192 and α193; Kao et al., 1984) are alkylated by bromoacetylcholine and therefore unable to reform disulfide bonds in the presence of DTNB, the cysteines near the binding site for mAbs 258 and 259 (α128 and α142) can re-form disulfide bonds in the presence of DTNB which permit these mAbs to bind as well as they had to untreated receptor. Therefore, it is very unlikely that in native receptor there are double disulfide bonds involving cysteines-α128 and -α142 linked to cysteines-α192 and -α193.

Asparagine-α141 is the only potential site for N-glycosylation in the α subunit (Noda et al., 1982). Therefore, we tested whether binding of concanavalin A could affect the

binding of mAbs to α127–143. As shown in Figure 6, preincubation of the immobilized receptor with this lectin prevented the binding of mAbs 258 and 259. Binding of concanavalin A to immobilized purified α subunits also inhibited binding of mAbs 258 and 259 (93% and 92%, respectively). By contrast, concanavalin A did not block the binding of mAbs to another site on the extracellular surface of α subunits, the main immunogenic region (data not shown). We were unable to observe the reversion of this effect by methyl mannoside because, for unknown reasons, the bound receptor dissociated from the wells after incubation with this compound. These results suggest that α141 is glycosylated and that mannoside residues are present and available for the lectin to bind. These results are consistent with the in vitro mutagenesis experiments of Mishina et al. (1985).

Added in Proof

Since this paper was submitted, Lennon et al. (1985) and Souroujon et al. (1985) have reported studies using similar synthetic peptides. Lennon et al. (1985) reported that immunization with α125–147 causes experimental autoimmune myasthenia gravis in rats. We did not notice this, nor did Souroujon et al. (1985) using α126–143 in rabbits, but mild muscular weakness might go unnoticed. Consistent with our results, Lennon et al. (1985) did not detect inhibition of α-bungarotoxin binding to receptor by anti-peptide antibodies. However, Lennon et al. (1985) reported that this peptide accounted for 26–56% of anti-receptor antibodies, which is not consistent with the observations we report here, nor the observations we report in Ratnam et al. (1986a), nor with Souroujon et al. (1985). Neither is this conclusion consistent with their own results, since in rats immunized with α125–147, Lennon et al. (1985) observed the same nanomolar range of anti-receptor titers which we observe, which is about 3 orders of magnitude less than the titers obtained after immunization with corresponding amounts of native receptor. They reported that excess ^{125}I-labeled anti-receptor antibodies added to an unspecified amount of peptide–Sepharose conjugate resulted in binding of 26–56% as many counts as when an unspecified amount of receptor–Sepharose conjugate was used. A meaningful experiment would be to measure the maximum amount of anti-receptor titer which could be adsorbed from anti-receptor sera by the peptide–Sepharose conjugate. Since this paper was submitted, Kao and Karlin (1986) have reported in an abstract that separate CNBr fragments of unreduced α subunits can be isolated, one containing disulfide-bonded cysteines-α128 and -α142 and another containing disulfide-bonded cysteines-α192 and -α193. This is consistent with the data presented here.

Acknowledgments

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Registry No. α127–143, 101494-08-0.

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Bovine Steroid 21-Hydroxylase: Regulation of Biosynthesis†

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ABSTRACT: A recombinant cDNA clone, PBC21-1, specific for bovine steroid 21-hydroxylase cytochrome P-450 (P-450$_{C21}$) was identified in a bovine adrenocortical cDNA library, and this identity was confirmed by nucleotide sequencing which revealed significant amino acid homology (77%) with human P-450$_{C21}$ cDNA. The pBC21-1 insert is 1.7 kilobases in length and includes a 1128 base pair region that encodes the C-terminal 376 amino acids of bovine P-450$_{C21}$ as well as 535 base pairs of 3′-untranslated sequence. A novel feature of this insert is a 20 base pair intervening sequence near the 5′ end, apparently the result of an aberrant splicing event. Northern blot analysis reveals that bovine P-450$_{C21}$ is encoded by two transcripts, 2.3 and 2.0 kilobases in length which are detected in adrenal cortical RNA. Bovine liver, heart, kidney, and corpus luteum do not contain detectable P-450$_{C21}$ transcripts. Regulation of P-450$_{C21}$ gene expression by adrenocorticotropin was investigated with pBC21-1 and bovine adrenocortical cells in primary, monolayer culture. Treatment with ACTH or analogues of cAMP increases the steady-state levels of P-450$_{C21}$ RNA in such cell cultures. In vitro transcription run-on assays suggest that this increase is, at least in part, due to the enhanced transcriptional activity of the P-450$_{C21}$ gene.

Biosynthesis of a number of physiologically active compounds such as glucocorticoids, mineralocorticoids, sex hormones, 1,25-dihydroxycholecalciferol, and bile acids requires mixed-function oxidase activity, catalyzed by various forms of cytochrome P-450 (Waterman et al., 1986). The particular forms of cytochrome P-450 localized in steroidogenic tissues (i.e., adrenal cortex, ovary and testis) are under both acute and chronic regulation by peptide hormones [either adrenocorticotropin (ACTH),[1] follicle-stimulating hormone (FSH), or luteinizing hormone (LH)], which thereby control the synthesis of steroid hormones, which in turn regulate a wide variety of metabolic and biosynthetic activities. In the adrenal cortex two mitochondrial forms of cytochrome P-450, cholesterol side-chain cleavage cytochrome P-450 (P-450$_{scc}$) and 11β-hydroxylase cytochrome P-450 (P-450$_{11\beta}$), and two microsomal forms of cytochrome P-450, 17α-hydroxylase cytochrome P-450 (P-450$_{17\alpha}$) and 21-hydroxylase cytochrome P-450 (P-450$_{C21}$), are components of the steroidogenic pathway

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[1] Abbreviations: P-450$_{C21}$, cytochrome P-450 specific for steroid 21-hydroxylation; P-450$_{scc}$, cytochrome P-450 specific for side-chain cleavage reaction of cholesterol; P-450$_{17\alpha}$, cytochrome P-450 specific for steroid 17α-hydroxylation; P-450$_{11\beta}$, cytochrome P-450 specific for steroid 11β-hydroxylation; SDS, sodium dodecyl sulfate; SSC, 1 × SSC = 0.15 M NaCl/15 mM sodium citrate; DTT, dithiothreitol; ACTH, adrenocorticotropin; RNase, ribonuclease; bp, base pair; kb, kilobase.

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