Structural Heterogeneity of the α Subunits of the Nicotinic Acetylcholine Receptor in Relation to Agonist Affinity Alkylation and Antagonist Binding†

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ABSTRACT: The structural basis for the heterogeneity of the two agonist binding sites of the *Torpedo californica* acetylcholine receptor with respect to antagonist binding and reactivity toward affinity alkylating reagents was investigated. There is one agonist binding site on each of the two α subunits in a receptor monomer. One of these sites is easily affinity labeled with bromoacetylcholine, while more extreme conditions are required to label the other. Evidence is presented that the site which is easily labeled with bromoacetylcholine is the site with higher affinity for the antagonist *d*-tubocurarine. Digestion of purified α subunits with staphylococcal V8 protease gave two limit fragments with apparent molecular weights of 17K and 19K. Both of these fragments began at residue 46 of the α sequence, and both reacted with monoclonal antibodies specific for the sequence α152–159 but not with antibodies specific for α235–242. Their tryptic peptide maps and reactivity with a number of monoclonal antibodies were virtually identical. Only the 17-kilodalton (17-kDa) fragments stained heavily for sugars with Schiff's reagent. However, both fragments bound ^{125}I-labeled concanavalin A. Complete removal of carbohydrate detectable with concanavalin A from V8 protease digests of α subunits resulted in two fragments of lower apparent molecular weights, indicating that these fragments differed not only in carbohydrate content but also in their C-termini or by another covalent modification. Covalent labeling of one of the two agonist sites of the intact receptor with bromo[^{3}H]acetylcholine followed by digestion with V8 protease resulted in labeling of only the 19-kDa fragment. When both agonist sites were affinity labeled, the 17-kDa fragment was also labeled. This suggests that the 19-kDa fragment is derived from one α subunit and the 17-kDa fragment from the other. It appears that while both α subunits of the receptor are glycosylated, one of the subunits is more heavily glycosylated than the other. The less glycosylated subunit contains the site of high reactivity with affinity alkylating reagents such as bromoacetylcholine and also the site with high affinity for *d*-tubocurarine, while the more heavily glycosylated subunit contains the site of low reactivity with these affinity reagents, which is also the site with low affinity for antagonists. The two α subunits might also differ by other covalent modifications.

The function of the nicotinic acetylcholine receptor is to permit the flow of cations across the postsynaptic membrane of skeletal muscles and fish electric organs in response to acetylcholine released from the nerve ending (Neher & Sakmann, 1976; Steinbach & Stevens, 1976). The receptor consists of four types of homologous, glycosylated polypeptides in the ratio $\alpha_2\beta\gamma\delta$ (Reynolds & Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980). Two molecules of agonist are thought to be required to bind to the receptor in order to elicit opening of its cation channel (Karlin, 1967; Changeux & Podleski, 1968; Dionne et al., 1978; Neubig & Cohen, 1980; Sine & Taylor, 1980; Hess et al., 1982). A number of agonists, antagonists, and elapid α toxins have been found to bind in a mutually exclusive fashion to the α subunit of the receptor (Moore & Raftery, 1979; Weiland et al., 1979; Hamilton et al., 1978; Neubig & Cohen, 1979). Systematic kinetic studies of the binding of these ligands have revealed that while the two agonist binding sites interact with positive cooperativity with respect to agonist binding, they behave as independent sites with different affinities for antagonists (Neubig & Cohen, 1979; Sine & Taylor, 1981; Culver et al., 1984). These ligands range in selectivity for one of the two sites, from compounds that show no selectivity (e.g., alcuronium) to those that show as much as a 300-fold difference in affinity (e.g., *d*-tubocurarine) (Neubig & Cohen, 1979). α-Bungarotoxin, on the other hand, does not differentiate between the two sites either in affinity or in rate of binding and binds with very high affinity ($K_d \sim 10^{-11}$ M) (Weber & Changeux, 1974a,b; Lee, 1979). Sulfhydryl affinity labeling reagents, e.g., bromoacetylcholine (BAC)[1] and [4-(*N*-maleimido)benzyl]trimethylammonium iodide (MBTA), have been used to label the acetylcholine binding site of the receptor. While it is

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[1] Abbreviations: BAC, bromoacetylcholine; Con A, concanavalin A; DPT, diaminophenyl thioether; DTT, dithiothreitol; mAb, monoclonal antibody; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium iodide; PBS, phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.5, and 100 mM NaCl); SDS, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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possible to easily label one site on the receptor (Damle et al., 1978; Karlin, 1980; Wolosin et al., 1980), labeling of the second site is more difficult and requires high concentrations of reducing agent and of the affinity reagent (Wolosin et al., 1980; Walker et al., 1984).

It has not been clear whether the difference in ligand binding between the two α subunits is a result of their assuming slightly different conformations due to the asymmetric subunit environment which they must experience as the result of the $\alpha_2\beta\gamma\delta$ subunit stoichiometry or whether there are covalent differences between the two α subunits. There is also a developmentally regulated difference between the two ligand binding sites which can be detected with antibodies (Gu et al., 1985), but the structural feature detected by this antibody is not known. The α subunits are glycosylated (Vandlen et al., 1979; Lindstrom et al., 1979). The two α subunits are thought to differ in their carbohydrate content, at least qualitatively (Lindstrom et al., 1983; Conti-Tronconi et al., 1984). Other covalent modifications of receptor α subunits which have been reported are phosphorylation (Vandlen et al., 1979; Davis et al., 1982) and covalent attachment of fatty acids (Olson et al., 1984).

Peptide maps of the purified α subunit obtained by digestion with staphylococcal V8 protease gave two limit fragments of molecular weights 17 000 and 19 000 (Gullick et al., 1981), both of which had the same N-terminal sequence while one fragment (17 kDa) had most of the carbohydrate (Lindstrom et al., 1983, Conti-Tronconi et al., 1984). These two fragments presumably come from two different α subunits. Gullick et al. (1981), from our laboratory, had earlier reported peptide mapping studies of the α subunit with V8 protease in which these two fragments were found to be exactly alike in their binding of a number of monoclonal antibodies (mAbs), while only the 19-kDa fragment was labeled with MBTA under conditions of single-site labeling. In this paper, we report the purification, mAb binding properties, concanavalin A (Con A) binding, and the N-terminal sequence of the two peptides and demonstrate the labeling of one or both of these fragments with [^{3}H]BAC under conditions of labeling of one or both agonist sites on the receptor. We further present evidence that the sites of low and high reactivity of the receptor with affinity alkylating reagents in fact correspond to the low- and high-affinity sites, respectively, for the binding of the reversible antagonist *d*-tubocurarine.

Experimental Procedures

Acetylcholine receptor from the electric organ of *Torpedo californica* was purified according to Lindstrom et al. (1978). Subunits of the receptor were purified by preparative SDS–polyacrylamide gel electrophoresis (Lindstrom et al., 1979). Native acetylcholine receptor rich membrane vesicles were purified by sucrose density gradient centrifugation (Elliott et al., 1980). Nonreceptor proteins were extracted from these vesicles by alkali treatment (Neubig et al., 1979).

Iodination of α-bungarotoxin to a specific activity of ~6 × 10^{17} cpm/mol was done with chloramine T (Lindstrom et al., 1981). Con A was iodinated similarly after its active site was protected with α-methyl-D-mannoside (Burridge, 1978).

BAC was synthesized by the method of Chiou and Sastry (1968). [^{3}H]BAC, bromide salt, was prepared as reported by Damle et al. (1978).

Proteolysis of the purified α subunit (10 μg) with V8 protease (Miles) (0.25–2.5 μg) was carried out in 40 μL of 10 mM sodium phosphate buffer (pH 7.4)/0.1% SDS for 1 h at room temperature. This solution was then mixed with an equal volume of 125 mM Tris-HCl buffer, pH 6.8, containing 0.1% SDS, 10% (v/v) glycerol, and 0.005% (w/v) bromophenol blue, heated at 100 °C for 5 min, and electrophoresed on a 15% SDS–polyacrylamide gel. For preparation of the fragments, 827 μg of the subunit was digested with 82.7 μg of protease, treated as above, and run on a 0.5-cm-thick gel. The separated fragments in the gel were located by brief staining and destaining (Cleveland et al., 1977). The appropriate subunit fragments were identified by comparison with a calibrated analytical gel. Individual fragment bands were purified by electroelution.

For amino acid sequencing, protein (1.5 nmol) was subjected to Edman degradation in the presence of 4–6 mg of polybrene in an automatic spinning cup sequencer with a 0.33 M Quadral single-coupling single-cleavage program (Spiess et al., 1982).

Protein blotting from electrophoretic gels was done on diaminophenyl thioether paper prepared by the method of Seed (1982). Probing of the blots with various antibodies was done essentially as described earlier (Gullick & Lindstrom, 1983). The binding of ^{125}I-Con A (1 nM) to blots was in 10 mM sodium phosphate (pH 7.5)/150 mM NaCl/10 mM NaN_3/1 mM $MnCl_2$/1 mM $CaCl_2$ for 2 h at 22 °C, followed by brief washing in the same buffer.

For deglycosylation of V8-proteolyzed α subunit, endoglycosidases F (NEN) and H (Miles) were used. Purified α subunit (2.5 μg of 0.5 mg/mL) as digested with V8 protease (0.63 μg) in the presence of 0.6% Triton X-100. After 1 h at room temperature, the protease was inactivated by boiling for 5 min in 1.7% SDS. Then two 6-μl aliquots (~1.1 μg of α subunit each) were removed. To one of the aliquots was added 28 μL of 50 mM EDTA, 60.8 mM DTT, and 100 mM sodium phosphate buffer, pH 6.1. This was divided into two 15.2-μL aliquots. One was treated with 0.81 unit (3.25 μL) of endoglycosidase F in 50% glycerol for 16 h at 37 °C, and the other was treated with 3.25 μL of 50% glycerol under the same conditions as a control. To the remaining 6-μL aliquot was added 24.5 μL of 68.7 mM DTT in 50 mM citrate buffer, pH 4.8. Two 13.4-μL aliquots were taken, and 5 μL of the same buffer alone (as control) or containing 10 milliunits of endoglycosidase H was added for 16 h at 37 °C.

Alkylation of the receptor in vesicles with BAC was carried out essentially as described by Wolosin et al. (1980) with some modifications. Alkali-extracted native receptor vesicles containing 2–5 μg of receptor were suspended in 1.0 mL of PBS containing 1.0 mM methanesulfonyl fluoride for 30 min at 22 °C to inhibit acetylcholinesterase activity. The vesicles were sedimented at 12800*g* for 30 min, and resuspended in 1 mL of 150 mM NaCl/4.5 mM NaN_3/1.5 mM EDTA/15 mM Tris-HCl, pH 8.0, and reduced with 0.3 mM DTT for 45 min at 22 °C. The pH was then adjusted to 7.0 with sodium dihydrogen phosphate, and 40-μL aliquots were added to 10 μL of the appropriate concentrations of BAC or 5 μM α-bungarotoxin followed by the addition of BAC. After 3 min at 22 °C, excess (~1 M) DTT was added to inactivate free BAC. The vesicles were diluted by adding 1 mL of PBS, and 5-μL aliquots were used for rapid immunoprecipitation assays of ^{125}I-α-bungarotoxin binding activity (Lindstrom et al., 1981). The vesicles were sedimented as before and dissolved in SDS sample buffer for electrophoresis or in 0.5% Triton/100 mM NaCl/10 mM sodium phosphate buffer, pH 7.5, for proteolysis with V8 protease.

The initial rate of binding of α-bungarotoxin to the receptor was measured by incubating receptor (solubilized or in native vesicles) equivalent to 3 nM toxin binding sites with 12 nM ^{125}I-α-bungarotoxin at 22 °C in *Torpedo* physiological saline (250 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM sodium phosphate buffer, pH 7, or in this buffer con-

Table I: Molecular Weights and Chemical and Antigenic Properties of Peptide Fragments of the α Subunit Obtained by Digestion with Staphylococcal V8 Protease[a]

fragment	app mol wt × 10^{-3}	mAb binding	special features
α	41	all	CHO, MBTA
V_A	39	6, 10, 13, 16, 42, 61, 65, 71, 203	CHO, MBTA
V_B	37	6, 10, 13, 16, 42, 61, 65, 71, 203	CHO, MBTA
V_C	34	6, 10, 13, 16, 42, 61, 65, 71, 203	CHO, MBTA
V_D	26	all	CHO, MBTA
V_E	19	6, 16, 42, 65, 71, 98, 203, 210, 237	MBTA
V_F	17	6, 16, 42, 65, 71, 98, 203, 210, 237	CHO
V_G	12	3, 5, 8, 19, 142, 149, 187	
V_H	10.5	3, 5, 8, 19, 142, 147, 149, 164, 187	
V_I	10	8, 147, 164	

[a] CHO denotes carbohydrate detected by Schiff staining; MBTA denotes labeling with [^{3}H]MBTA under conditions in which one of the acetylcholine binding sites is labeled.

taining 0.5% Triton X-100 for solubilized receptor). After 1 and 2 min, aliquots of 100 μL were removed into tubes containing 10 μL of 1 mg/mL cold α-bungarotoxin and vortexed immediately. The receptor was immunoprecipitated, and the ^{125}I-α-bungarotoxin bound was estimated by the usual rapid immunoprecipitation assay (Lindstrom et al., 1981). In a control experiment, it was ensured that the cold bungarotoxin did not displace ^{125}I-α-bungarotoxin (Lo et al., 1981). Under the above conditions, binding of ^{125}I-α-bungarotoxin at 2 min was roughly twice that at 1 min, indicating that the measured rates of toxin binding were true initial rates.

The binding of *d*-tubocurarine at various concentrations was monitored by measuring inhibition of the initial rate of toxin binding to the receptor produced by *d*-tubocurarine. In this experiment, receptor was preincubated for 1 h at 22 °C with various concentrations of *d*-tubocurarine and the initial rate of ^{125}I-α-bungarotoxin binding measured as above.

Results

Characterization of Peptide Fragments of the α Subunit Obtained with Staphylococcal V8 Protease. Table I summarizes the molecular weights and antigenic and chemical properties of the various fragments of the α subunit obtained by digestion with staphylococcal V8 protease. Of particular interest are the fragments V_E and V_F which are limit fragments and could not be further digested even at high enzyme/protein ratios. These fragments have apparent molecular weights on SDS–polyacrylamide gels of 19K and 17K, respectively. Both the fragments have very similar antigenic properties. In addition to previously published results (Gullick et al., 1981), a number of other antibodies in our library directed against the α subunit (mAbs 42, 65, 71, 98, 203, 210, and 237) bound to both of the fragments on Western blots. The two fragments also gave nearly identical two-dimensional tryptic peptide maps (results not shown). Only the fragment V_E could be labeled with the affinity alkylating reagent MBTA under conditions for labeling of a single agonist site on the receptor (Gullick et al., 1981). Fragment V_F could be stained much more intensely than V_E with Schiff's reagent (Gullick et al., 1981). However, ^{125}I-Con A bound to both the fragments on Western blots (Figure 1); this binding was specific to mannose residues, as the bound ^{125}I-Con A could be released by washing the blot with 1 M α-methyl-D-mannoside (Figure 1). Although the intensities of the radioactivity in the two bands in Figure 1

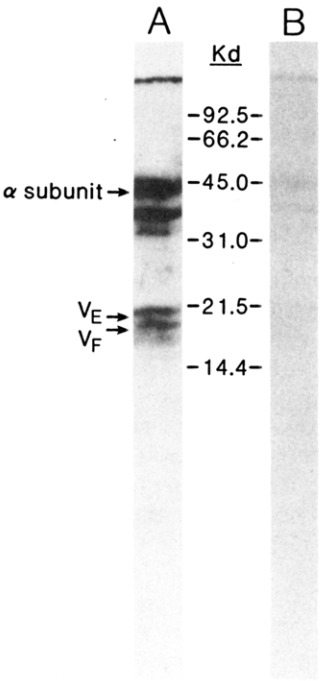

FIGURE 1: Binding of ^{125}I-Con A to proteolytic fragments of the α subunit. A partial staphylococcal V8 protease digest of the α subunit (10 μg) was electrophoresed on an SDS–15% polyacrylamide gel and transferred electrophoretically to DPT paper. The blot was probed with 1 nM ^{125}I-Con A followed by autoradiography. Lane A is the Western blot probed with ^{125}I-Con A. Lane B is the blot after specific elution of the bound Con A with 1 M methyl α-mannoside.

are roughly equal, they do not reflect the relative amounts of mannose residues present in them since a single molecule of Con A bound to a sugar side chain could preclude the binding of other Con A molecules at adjacent sites simply because of the large size of the Con A tetramer.

In view of the close similarity of the fragments V_E and V_F and some interesting differences, the two fragments were purified and their amino acid compositions and N-terminal sequences determined. The N-terminal sequences of the fragments were identical and had the sequence *N*-Val-Asn-Gln-Ile-Val-X-Thr-Asn-Val-Arg-Leu, which corresponds to residues 46–56 of the α-subunit sequence (Noda et al., 1982). Both of these peptides bound mAb 237, which is specific for the sequence α152–159 (Criado et al., 1985a), so their C-termini must be beyond α159. Subsequent results will show that both peptides are also labeled with [^{3}H]BAC, so their C-termini must also be beyond α192, where this reagent probably reacts (Kao et al., 1984). Neither of these fragments bound mAb 254, which is specific for the sequence α235–242 (Criado et al., 1985b), indicating that the C-termini of both V_E and V_F precede the residue α242.

We tested the possibility that V_E and V_F had identical amino acid sequences and differed only in the amount of carbohydrate they contained. If this were so, then complete removal of carbohydrate from V_E and V_F should result in a single V_8 protease fragment. Figure 2 shows that both endoglycosidase H and endoglycosidase F were capable of removing all carbohydrate detectable by binding of ^{125}I-Con A. However, instead of V_E and V_F being converted to a single band by removal of carbohydrate, two bands of lower apparent molecular weights were observed. Therefore, either V_E and V_F differ somewhat in their C-termini (e.g., V_F terminates after aspartic acids-α195 or -200 or V_E terminates after aspartic acid-α238, which would account for a molecular weight difference of 4370–4945) or V_E and V_F differ by another covalent

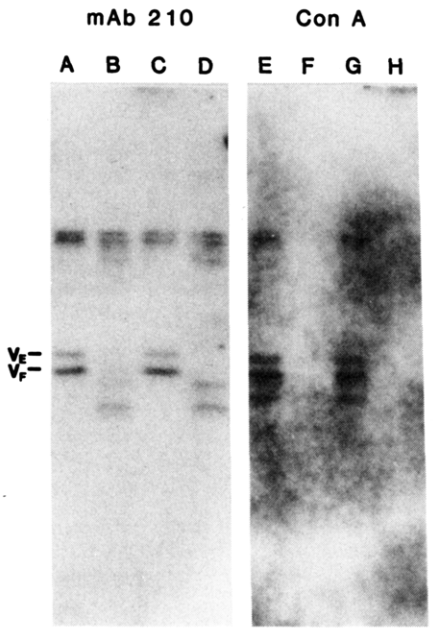

FIGURE 2: Treatment of V8 protease digested α-subunit peptide fragments with endoglycosidases F and H. The endoglycosidase-treated and control peptide samples were resolved by electrophoresis on a 15% acrylamide gel and then electroblotted onto DPT paper. Half of the blot (lanes A–D) was exposed to mAb 210 followed by ^{125}I-labeled goat anti-rat IgG prior to autoradiography in order to localize V_E, V_F, and other peptide fragments. The other half of the blot containing the same series of sample (lanes E–H) was probed with ^{125}I-Con A to localize those peptides which contained carbohydrate. Lanes A, C, E, and G are control V8 protease digests subjected to the same incubation conditions as the adjacent endoglycosidase-treated samples. Lanes B and F were treated with endoglycosidase F. Lanes D and H were treated with endoglycosidase H.

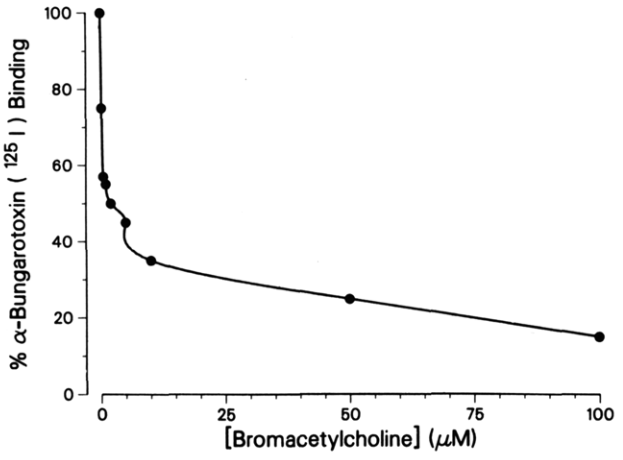

FIGURE 3: Inhibition of the equilibrium binding of ^{125}I-α-bungarotoxin to receptor in alkali-treated vesicles after alkylating reduced receptors with various concentrations of BAC.

posttranslational modification such as covalent attachment of lipid (Olson et al., 1984).

Affinity Labeling of the Receptor with BAC. Figure 3 shows the pattern of inhibition of α-bungarotoxin binding to alkali-treated native receptor vesicles after alkylation with various concentrations of BAC. There are apparently two phases of inhibition as reported by Wolosin et al. (1980) with about 50% inhibition being achieved in the concentration range 5–10 μM BAC. Nearly complete (85%) inhibition of toxin binding was achieved at about 100 μM BAC.

Labeling of V_E and V_F with [^{3}H]BAC. Since affinity labeling of the α subunits of the receptor with [^{3}H]BAC required whole receptor, it was necessary to establish the identity of

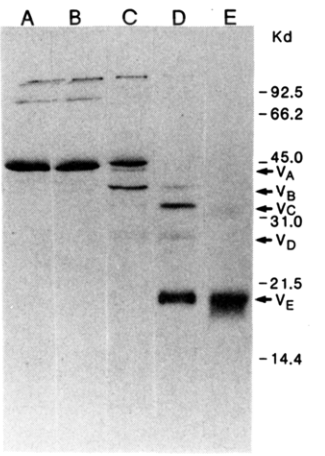

FIGURE 4: Digestion of [^{3}H]BAC-labeled, intact acetylcholine receptor with various concentrations of staphylococcal V8 protease. Receptor (5 μg) labeled at one site with [^{3}H]BAC was digested with 0.0025 (lane B; lanes labeled A to E from left to right), 0.25 (lane c), 0.25 (lane D), or 2.5 μg (lane E) V8 protease. A control sample (lane A) was not treated with enzyme. The samples were run on an SDS–15% polyacrylamide gel followed by fluorography using Enhance (New England Nuclear).

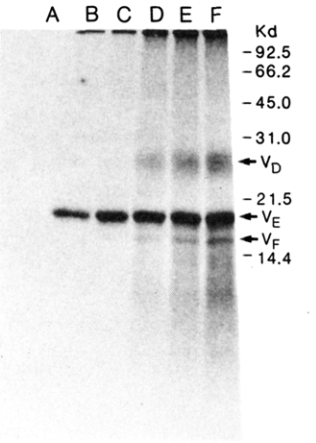

FIGURE 5: Labeling of fragments V_E and V_F after alkylation of the receptor with various concentrations of [^{3}H]BAC. Lane A, control, preincubated with 5 μM cold α-bungarotoxin, followed by 100 μM [^{3}H]BAC. Lanes B, C, D, E, and F, receptor treated with 0.2, 0.5, 10, 50, and 100 μM [^{3}H]BAC, respectively. The alkylated samples were digested with 2.5 μg of V8 protease, electrophoresed on an SDS–15% polyacrylamide gel, and fluorographed with Enhance (New England Nuclear). The extent of alkylation of samples in lanes B, C, D, E, and F corresponded respectively to 75, 60, 35, 25, and 15% residual α-bungarotoxin binding activity of the receptor.

the fragments of the α subunit obtained by V8 protease digestion of whole receptor with the proteolytic fragments of the purified α subunit. Figure 4 shows a fluorogram of the peptide map of the intact receptor that was labeled at one site with [^{3}H]BAC. Fragments arising from the α subunit can be identified by the presence of the radioactive label. All the fragments with molecular weights corresponding to those of V_A–V_E were labeled with [^{3}H]BAC.

At low concentrations of [^{3}H]BAC (0–5 μM), only the fragment V_E was labeled, whereas at higher concentrations (10–100 μM) the label appeared in fragment V_F as well, with increasing intensity (Figure 5). The labeling of these fragments was specific, as preincubation with cold α-bungarotoxin resulted in the absence of radioactivity (lane A in Figure 5). The intensity of radioactivity in V_F, however, was lower than in V_E, and this is likely to be due to incomplete alkylation, incomplete proteolysis, and aggregation. It can be seen that V_D is labeled in parallel with V_F. It may, therefore, be a

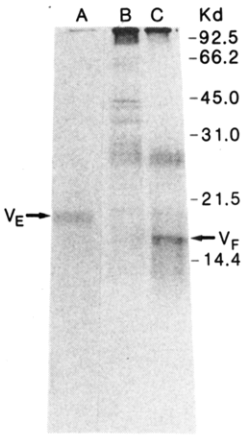

FIGURE 6: Selective labeling of fragment V_F by [^{3}H]BAC. Receptor was labeled with 0.2 μM [^{3}H]BAC (lane A) or preincubated with 5 μM cold BAC followed by treatment with 100 μM [^{3}H]BAC (lanes B and C). The alkylated samples were digested with 0.25 μg of V8 protease (lane B) or with 2.5 μg of V8 protease (lanes A and C).

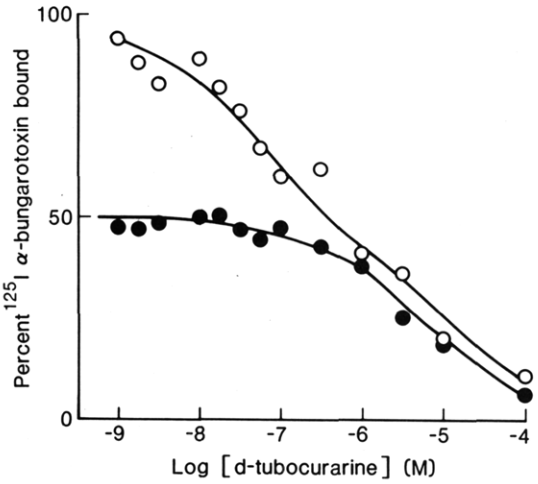

FIGURE 7: Inhibition of the initial rate of ^{125}I-α-bungarotoxin binding to receptor in native vesicles (○) or the receptor alkylated at 50% of the toxin binding sites with BAC (●).

precursor of V_F. V_D probably extends beyond V_F to very near the C-terminus of the α subunit, since Table I shows that V_D binds mAbs 3, 5, and 8, whose binding sites are in the sequence α349–372 (Ratnam et al., 1986). The specificity of the labeling of V_E and V_F with [^{3}H]BAC was further confirmed by preincubating the receptor with a low concentration (5 μM) of unlabeled BAC, followed by treatment with a high concentration (100 μM) of [^{3}H]BAC. In this case, fragment V_F was selectively labeled (Figure 6).

Binding of d-Tubocurarine to α-Bungarotoxin-Labeled Receptor. Since the antagonist *d*-tubocurarine bound with two apparently independent affinities to the membrane-bound receptor (Neubig & Cohen, 1979), it was of interest to examine the affinity of *d*-tubocurarine to the receptor blocked with BAC at the site of high BAC reactivity. The binding of *d*-tubocurarine at various concentrations was measured by its ability to reduce the initial rate of binding of α-bungarotoxin. In the case of native receptor, the antagonist produced inhibition of toxin binding over a wide range of concentrations of *d*-tubocurarine (10^{-9}–10^{-5} M), with about 50% inhibition being achieved at about 10^{-6} M (Figure 7). In the case of the receptor labeled with BAC at the highly reactive site, the curare binding pattern was shifted significantly to higher concentration of the antagonist and occurred over a narrower range of curare concentrations (Figure 7), suggesting that the acetylcholine binding site which remained after 50% labeling

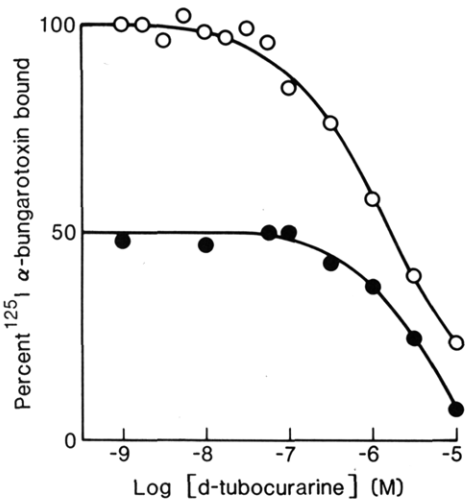

FIGURE 8: Inhibition of the initial rate of ^{125}I-α-bungarotoxin binding to receptor solubilized in 0.5% Triton X-100 (○) or the solubilized receptor labeled at 50% of the toxin binding sites with BAC (●).

with BAC was the site with low affinity for curare.

Solubilization of the receptor from native vesicles in 0.5% Triton X-100 resulted in shifting of the *d*-tubocurarine binding curve to higher concentrations of the antagonist (Figure 8). These results show that detergent solubilization of the receptor decreases its affinity for *d*-tubocurarine. In the case of receptor that was labeled at 50% of the agonist binding sites with BAC before solubilization, there was a further slight shift of the binding curve to higher concentrations of *d*-tubocurarine (Figure 8). This also suggests that the site with low reactivity for BAC is the site with low affinity for curare.

Discussion

There is substantial evidence from sequencing studies (Raftery et al., 1980; Noda et al., 1982, 1983a,b) as well as by synthesis of functional receptor from cloned cDNA (Mishina et al., 1984) that the two α subunits of the nicotinic acetylcholine receptor have identical amino acid sequences. In *Torpedo*, only a single gene codes for α subunits (Klarsfeld et al., 1984). Ligand binding domains on the α subunit would thus be expected to be identical. The observed differences in ligand binding properties of the two subunits could then be attributed to subtle differences in structure caused by (1) conformational asymmetry in the environments of the two α subunits within the quaternary structure of the receptor or (2) chemical differences caused by differential posttranslational modifications. These two factors are of course not mutually exclusive, and, further, conformational differences could be the basis for different posttranslational modifications of the two α subunits.

The two fragments V_E (19 kDa) and V_F (17 kDa) in peptide maps of the purified α subunit (Table I) obtained by digestion with staphylococcal V8 protease must come from different populations of α subunits. This is supported by the following facts: (1) They can be differentially labeled under conditions that differentially label the two acetylcholine binding sites of receptor; (2) both these fragments are limit digests, and the 19-kDa fragment cannot be digested further to give the 17-kDa fragment; (3) both the fragments have the same N-terminus but may differ slightly in C-terminus or in covalent modification in addition to glycosylation; and (4) the fragments differ in glycosylation (Gullick et al., 1981; Conti-Tronconi et al., 1984). Both the fragments bind mAb 237 on Western blots (Table I). This antibody is specific for the sequence 152–159 (Criado et al., 1985a). The only possible site for N-linked

glycosylation on the α subunit is the asparagine residue at position 141, which must be present in both fragments. Evidence suggests that the α subunit is glycosylated at only one site (Anderson et al., 1981; Merlie et al., 1981) and that this site is α141 (Mishina et al., 1985; Criado et al., 1986). It is not surprising then that fragment V_E also has some amount of glycosylation as revealed by the ability of ^{125}I-Con A to bind to it on Western blots (Figure 1). Consistent with this finding, Con A also inhibited α-bungarotoxin binding to the receptor to the same extent, in the case of both unlabeled receptor and receptor labeled at one site with BAC (results not given). In muscle cells, α subunits are cotranslationally glycosylated (Merlie et al., 1982). It seems likely that differences in glycosylation between the two α subunits would occur posttranslationally due to the addition and/or subtraction of some sugars.

The actual lengths of the fragments of the α subunit cannot be simply predicted from their apparent molecular weights on SDS gels as the denatured α subunit displays anomalous mobilities on these gels (Ratnam & Lindstrom, 1984). The C-terminus of fragments V_E and V_F should lie on the amino-terminal side of α242, as mAb 254, which is specific for α235–242 (Criado et al., 1985b), does not bind to either fragment on Western blots. Further, it has been shown (Kao et al., 1984) that the sites of alkylation with agonist affinity alkylating reagents on the α subunit are cysteine residues at positions 192 and 193. Since both V_E and V_F can be alkylated specifically with BAC, the C-termini of these fragments must occur beyond residue 192. Staphylococcal V8 protease is specific for peptide bonds at the carboxyl groups of aspartic and glutamic acids, under the conditions employed here [reviewed by Drapeau (1977)]. Inspection of the reported amino acid sequence of the α subunit (Noda et al., 1982) shows that there are only three possible V8 protease cleavage sites between residues 193 and 242 and these are the aspartic acid residues at positions 195, 200, and 238. V_E and V_F, thus, have C-termini which either are identical or differ by 5–43 residues. Because removal of carbohydrate (Figure 2) did not abolish the apparent molecular weight difference between fragments V_E and V_F, they must differ not only in glycosylation but also in either C-terminus and/or other covalent modifications such as covalent attachment of lipid (Olson et al., 1985). Because approximately equal amounts of V_E and V_F are produced from the two α subunits even after denaturation of the subunits of SDS, the differences between these fragments do not result from different α conformations in native receptor but from intrinsic covalent differences in the α subunits. If the difference in glycosylation between the two α subunits is the only covalent difference, it must cause a difference in the site of C-terminal cleavage of V_E and V_F, if there is a difference, or there must be an additional covalent difference between the two subunits. It is relevant to note here that there is considerable variability in the pattern and N-terminal sequences reported by various groups for V8 protease generated fragments of α subunits in the range 17–21 kDa [examples in Conti-Tronconi et al. (1984), Neumann et al. (1985), Wilson et al (1985), and Pederson et al. (1986)]. We have observed that this is due to the extreme sensitivity of the cleavage specificity of V8 protease to conditions of pH and detergent concentration (results not shown).

The agonist affinity alkylating reagents BAC and MBTA can easily label only one of the two agonist binding sites of the receptor (Damle et al., 1978; Karlin, 1980; Wolosin et al., 1980). Labeling of the second site requires a high concentration of DTT in order to prevent rapid reoxidation of reduced cysteines and also a very high concentration of the alkylating agent (Wolosin et al., 1980). The latter requirement reflects either (1) a lower affinity of the second site for the initial binding of the agonist or (2) a lower reactivity of the sulfhydryl group at this site with reducing and/or alkylating reagents. In order to identify the α subunits that contain the sites of high or low reactivity with BAC, the fragments V_E and V_F were analyzed for the presence of radioactivity after specifically labeling the intact receptor at either one or both sites with [^{3}H]BAC (Figures 3–6). These experiments clearly showed that the sites of high and low reactivity with BAC lie within the fragments V_E and V_F, respectively.

Table II: Different Properties of the Two α Subunits of Acetylcholine Receptor

	α_1	α_2
glycosylation	low	high
BAC reactivity	high	low
curare affinity	high	low
characteristic peptide fragments	V_E	V_D, V_F

A number of antagonists bind to the acetylcholine receptor with apparent negative cooperativity (Neubig & Cohen, 1979). The possibility of the presence of two interacting antagonist binding sites where both have the same initial affinity for the binding of the first antagonist molecule is very unlikely in light of kinetic studies of the binding of antagonist to receptor in states of partial occupancy by agonists or α-bungarotoxin (Neubig & Cohen, 1979). The pattern of inhibition of the initial rates of α-bungarotoxin binding to the receptor by the antagonist *d*-tubocurarine (Figure 7) gives a good qualitative indication of the presence of separate high- and low-affinity sites for the binding of antagonists (Neubig & Cohen, 1979). Using this criterion, it was found that alkylation of the receptor with BAC at the site of high reactivity for the reagent abolished the high-affinity site of *d*-tubocurarine binding (Figure 7), indicating that these two sites occur on the same α subunit. Further, from the results described earlier, this subunit, α_1, as described in Table II, is the less glycosylated α subunit. Gu et al. (1985) have reported an autoantibody from a myasthenia gravis patient which blocks the site with high affinity for curare. It is especially interesting that this antibody (which can also bind to receptors from *Torpedo*) binds only to an early developmental form of receptor. This would be consistent with the idea that this developmental form of receptor differed in glycosylation or other posttranslational modification from latter developmental forms at α_1.

The structural differences at the two agonist binding sites of the receptor are apparently very subtle, as acetylcholine can bind to both the subunits with the same initial affinity (Neubig & Cohen, 1979; Sine & Taylor, 1980; Steinbach, 1980). Further, antagonists show decreasing differences in affinity for the two sites in the order of increasing affinities of these compounds, an extreme case being α-bungarotoxin which is an antagonist of very high affinity that does not distinguish between the two sites (Neubig & Cohen, 1979).

It is interesting that solubilization of the receptor with Triton X-100 disrupts the high-affinity binding site for *d*-tubocurarine (Figure 8). In this case, previous blocking of the high-affinity sites with BAC does not appreciably alter the pattern of *d*-tubocurarine binding (Figure 8). Detergent solubilization has previously been reported to decrease the affinity of agonist binding to the receptor (Sugiyama & Changeux, 1975; Chang & Bock, 1979). This effect thus seems to apply to antagonist binding, as well.

Although we have clear evidence that the two α subunits of receptor differ in glycosylation and ligand binding properties

(summarized in Table II), we cannot now determine whether the differences in ligand binding are caused directly by glycosylation. The low pH optima of endoglycosidases required to remove carbohydrate have foiled our attempts to remove carbohydrate from native receptor. Observation that prevention of glycosylation impairs subunit assembly (Merlie et al., 1983) also complicates interpretation of in vitro mutagenesis experiments (Mishina et al., 1985). It could as well be that the differences in both their glycosylation and their ligand binding affinities are the result of different conformations of the two α subunits due to the different subunits which surround them in the asymmetric $\alpha_2\beta\gamma\delta$ structure of the receptor monomer. About 30 min is required after the syntheses of α subunits before they are assembled into receptors (Merlie et al., 1983), and during this time, they undergo some conformational maturation (Merlie & Lindstrom, 1984). It may be critical to establish whether the asymmetric glycosylation of α subunits occurs before or after their assembly with the other receptor subunits. Also, it may be critical to determine whether some other covalent modification accounts for the functional and structural differences between the two α subunits.

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Registry No. BAC, 17139-54-7; *d*-tubocurarine, 57-95-4.

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Structural Characterization of the ATP-Hydrolyzing Portion of the Coated Vesicle Proton Pump†

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ABSTRACT: The ATP-hydrolyzing portion of the proton pump from clathrin-coated vesicles (isolated from calf brain) was solubilized with three nondenaturing detergents (cholate, octyl glucoside, and Triton X-100). The hydrodynamic properties of the solubilized (Mg^{2+})-ATPase were then determined by sedimentation analysis in H_2O and D_2O and gel filtration on Sepharose 4B. The coated vesicle (Mg^{2+})-ATPase migrated under all conditions as a single peak of activity. In cholate, the sedimentation coefficient ($s_{20,w}$), Stokes radius (a), and partial specific volume ($\bar{v}_c$) were 8.25 (±0.20) S, 68 (±2) Å, and 0.71 (±0.03) cm^3/g, respectively. In octyl glucoside and Triton X-100 these values were respectively 7.90 (±0.20) and 7.45 (±0.20) S, 68 (±3) and 101 (±5) Å, and 0.74 (±0.03) and 0.75 (±0.03) cm^3/g. Application of the Svedberg equation to these data gave a molecular weight for the protein–detergent complex of 217 000 ± 21 000 (cholate), 234 000 ± 26 000 (octyl glucoside), and 337 000 ± 40 000 (Triton X-100). Assuming the protein binds one micelle of detergent, these values correspond to a protein molecular weight of 215 000 ± 21 000 (cholate), 226 000 ± 26 000 (octyl glucoside), and 247 000 ± 40 000 (Triton X-100). The cholate-solubilized, gradient-purified (Mg^{2+})-ATPase, when combined with a 100000g pellet fraction, could be reconstituted by dialysis into phospholipid vesicles which displayed ATP-dependent proton uptake. The sensitivity of this acidification and the (Mg^{2+})-ATPase activity of the detergent-solubilized protein to 7-chloro-4-nitro-2,1,3-benzoxadiazole (NBD-Cl) and *N*-ethylmaleimide (NEM) and their resistance to ouabain are consistent with this protein complex corresponding to the ATP-hydrolyzing portion of the coated vesicle proton pump.

Results obtained in a variety of systems have suggested that exposure to low pH in a prelysosomal compartment is the signal which activates ligand–receptor dissociation following receptor-mediated endocytosis (Tycko & Maxfield, 1982; Merion & Sly, 1983; Marsh et al., 1983; Harford et al., 1983). Clathrin-coated vesicles have been shown to contain an ATP-dependent proton pump which has been postulated to be responsible for this acidification (Forgac et al., 1983; Stone et al., 1983). The coated vesicle pump is similar in inhibitor sensitivity and ionic requirements to a number of other intracellular proton pumps, including those present in endosomes (Galloway et al., 1983; Yamashiro et al., 1983), lysosomes (Schneider, 1981; Harikumar & Reeves, 1983), and Golgi-derived vesicles (Glickman et al., 1983; Zhang & Schneider, 1983), but its relationship to these pumps remains uncertain. We have also demonstrated that the coated vesicle pump shares a number of important properties with the mitochondrial (H^+)-ATPase, including the absence of a phosphorylated intermediate (Forgac & Cantley, 1984), although these proteins are distinguishable on the basis of their sensitivity to oligomycin and aurovertin (Forgac et al., 1983). Reconstitution

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