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Location of a Polypeptide Sequence within the α-Subunit of the Acetylcholine Receptor Containing the Cholinergic Binding Site

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SUMMARY

Proteolytic fragments of the α-subunit of the acetylcholine receptor of *Torpedo* electric organ were generated by digestion with *Staphylococcus aureus* V8 protease, and their ability to bind α-bungarotoxin was assessed following resolution on polyacrylamide gels and transfer to nitrocellulose. The position of the smallest fragment (M_r = 17,000) with toxin-binding activity was located within the primary sequence of the α-subunit by isolation and chemical characterization. The amino acid sequence at its amino terminus is Val-Asn-Gln-Ile-Val-Glu, which is identical to a unique sequence on the α-subunit beginning at Val 46. Based on considerations of the apparent molecular weight of this polypeptide fragment, the enzyme specificity of V8 protease, analysis of the partial amino acid composition, and the position of various identifying landmarks of the primary sequence of the α-subunit, the carboxy terminus is restricted to an acidic amino acid residue between Asp 152 and Asp 180. The apparent affinities of the 17-kDa polypeptide for α-bungarotoxin (IC_{50} = 100 nM) and *d*-tubocurarine (IC_{50} = 0.4 mM) were not significantly different from the values obtained for the undigested α-subunit, suggesting that the 17-kDa polypeptide contains all of the essential elements present on the α-subunit that are necessary for the binding of both of these cholinergic ligands. A second toxin-binding fragment (M_r = 19,000) was also identified. Although the amino terminus of this polypeptide fragment has not been determined directly, its position relative to Asn 141, the site of *N*-glycosylation, was established, and it appears that its amino terminus cannot be located any closer to the amino terminus of the α-subunit than Asp 152. If this tentative assignment is correct, then the region of the α-subunit involved in binding cholinergic ligands is more closely defined as occurring within the segment of the primary sequence of the α-subunit that the 17- and 19-kDa polypeptide fragments have in common, i.e., an overlapping sequence maximally bounded by Asp 152 and Asp 180.

The nicotinic AcCh receptor is a transmembrane protein complex that increases the membrane permeability to cations upon binding AcCh. The receptor from the electric organ of *Torpedo* has been well characterized structurally and has been shown to be composed of five polypeptide subunits with a molar stoichiometry of $\alpha_2 \beta \gamma \delta$ (1). The primary amino acid sequences of each of the subunits have been determined by recombinant DNA techniques (2-6) and analyzed to construct structural models of how the constituent polypeptides may be folded and assembled into an AcCh-regulated ion channel (4, 7-9). Identification of the location of the AcCh-binding site on the receptor is important information for understanding the molecular mechanism by which the binding of AcCh induces a receptor-conformational transition to an ion-transporting state (10). Previously we have demonstrated, utilizing a novel protein transfer-binding assay, that the cholinergic binding site resides on the α-subunit (11), a result that is in agreement with work by other laboratories (12, 13). In addition, we (14) and others (15, 16) have been able to identify proteolytically derived fragments of the α-subunit that are able to bind ^{125}I-α-BgTx. In this paper we report the isolation of a toxin-binding fragment of the α-subunit (M_r = 17,000) that was generated by digestion with *Staphylococcus aureus* V8 protease. The location of this fragment on the primary sequence of the α-subunit has been established, providing a definition of the region of the α-subunit that binds cholinergic ligands.

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Materials and Methods

AcCh receptor-enriched membranes from the electric organ of *Torpedo californica* were prepared according to the method of Sobel *et al.* (17). The specific activity of this preparation was 2-4 nmol of α-BgTx sites/mg of protein. ^{125}I-α-BgTx (specific activity 120 Ci/mmol) and ^{3}H-MBTA (specific activity 30 Ci/mmol) were purchased from New England Nuclear Corp. *Staphylococcus aureus* V8 protease (lot 0015) was obtained from Miles Laboratories and had an activity of 505 units/mg at pH 7.8 with casein as substrate. Endo F was a gift of H. M. Goodman (University of Massachusetts Medical School) who obtained it from New England Nuclear Research Products. The nitrocellulose

ABBREVIATIONS: AcCh, acetylcholine; α-BgTx, α-bungarotoxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium iodide; Endo F, endoglycosidase F; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin.

sheets, pore sizes 0.45 μm and 0.2 μm, were purchased from Schleicher and Schuell.

SDS-gel electrophoresis and enzymatic digestion of the α-subunit. The α-subunit was isolated from AcCh receptor-enriched membranes by preparative SDS-PAGE according to the method of Laemmli (18) with the exception that 2-mercaptoethanol was omitted from the sample buffer and no heating was performed. The positions of the resolved polypeptides were determined by placing the gel on a plastic sheet coated with silica gel impregnated with a fluorescent indicator (E. Merck) and illuminating with UV light of 254 nm. The resolved polypeptides absorb the UV light and are thus located as dark bands against a bright green fluorescent background. The region of the gel containing the α-subunit was excised from the gel, and the α-subunit was obtained by electroelution (19) and dialyzed overnight with enzyme incubation buffer (10 mM Tris, 10 mM NaCl, 0.001% SDS, pH 6.8).

Proteolytically derived fragments of the α-subunit were obtained by incubating the isolated α-subunit with varying amounts of *S. aureus* V8 protease for 60 min at room temperature. Enzymatic digestion was terminated by the addition of electrophoretic sample buffer lacking 2-mercaptoethanol. The resulting polypeptide fragments were resolved on a 15% SDS-polyacrylamide gel. Enzymatic digestion products of M_r = 17,000 and 19,000 were isolated by preparative SDS-PAGE using the same fluorescent detection and electroelution methods described above for the α-subunit.

Transfer to nitrocellulose sheets and ^{125}I-α-BgTx binding. Following equilibration of the SDS-polyacrylamide gels for 30 min in transfer buffer (24 mM Tris, 192 mM glycine, 0.001% SDS, pH 8.3), the proteolytically derived polypeptide fragments were electrophoretically transferred for 3 hr at 250 mamp to nitrocellulose sheets. The nitrocellulose sheet was then placed in prebinding buffer (20 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 7.0) supplemented with 4.0% BSA for 4 hr at 22°. This step reduces substantially the amount of labeled toxin that adsorbs to the nitrocellulose sheet. The sheet was then placed in a plastic pouch (Seal-a-Meal), containing 10 nM ^{125}I-α-BgTx in 20 mM NaCl, 1 mM EDTA, 10 mM Tris, and 1.0% BSA, pH 7.0. Following a 4-hr incubation at 22°, the sheets were washed 10 times for 1 min each with ice-cold wash buffer (200 mM NaCl, 1 mM EDTA, 10 mM Tris, and 0.02% BSA, pH 7.0), air dried, and exposed to Kodak X-Omat film (1–3 days). The apparent molecular weights of the radiolabeled bands were determined by the use of radiolabeled molecular weight standards: ^{125}I-nerve growth factor and ^{125}I-human growth hormone, 14,000 and 21,000, respectively, and by non-radiolabeled molecular weight standards: lysozyme (14,400), myoglobin (17,000), soy bean trypsin inhibitor (21,500), and carbonic anhydrase (31,000), which were identified by amido black staining after transfer to nitrocellulose.

To determine whether cholingeric ligands could compete with ^{125}I-α-BgTx for binding sites on the 17-kDa polypeptide, nitrocellulose strips containing equal amounts of the 17-kDa polypeptide were preincubated with 4% BSA and then incubated overnight with 3 nM ^{125}I-α-BgTx containing increasing concentrations of either *d*-tubocurarine or unlabeled α-BgTx. Following incubation the strips were rapidly washed, air dried, and exposed to Kodak X-Omat film (1–3 days). To quantitate the amount of ^{125}I-α-BgTx binding in the presence of cholinergic ligands, the area of the nitrocellulose strip containing the radiolabeled band was excised and the amount of radioactivity was determined by gamma emission. The background binding of ^{125}I-α-BgTx to the nitrocellulose strip alone was determined by removing an equivalent area not containing a radioactive band, and this value was subtracted from the total radioactivity.

Enzymatic digestion of the α-subunit labeled with ^{3}H-MBTA. AcCh receptor-enriched *Torpedo* membrane fragments were reduced with dithiothreitol and labeled covalently with ^{3}H-MBTA (final concentration 1 μM) according to the method of Karlin and Cowburn (20). The labeled α-subunit was isolated from the other receptor subunits by preparative SDS-PAGE and electroelution as described above. The radiolabeled α-subunit was incubated with V8 protease and the resulting polypeptide fragments were resolved on a 15% SDS-polyacrylamide gel and stained with Coomassie blue. To identify which of the fragments were radiolabeled, the gel was prepared for fluorography (21) and exposed to Kodak X-Omat film for 5 weeks at −80°.

Treatment of the isolated 17- and 19-kDa polypeptide fragments with Endo F. Isolated 17- and 19-kDa polypeptide fragments of the α-subunit were incubated with Endo F in 0.1 M sodium phosphate, pH 6.1, containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, and 1% 2-mercaptoethanol according to the procedure of Rosenzweig *et al.* (22).

Biochemical characterization of the 17-kDa polypeptide. The initial amino acid sequence at the amino terminus of the 17-kDa polypeptide fragment was determined by automated Edman degradation in a gas-phase sequencer (Applied Biosystems). The phenylthiohydantion derivatives of the amino acids were identified and quantitated using two independent reverse phase high pressure liquid chromatography systems.

A partial amino acid composition of the 17-kDa polypeptide was obtained following hydrolysis *in vacuo* with 6 N HCl at 110° for 24 hr. The hydrolyzate was analyzed spectrofluorometrically following precolumn derivatization using *o*-phthalaldehyde by the procedure of Jones *et al.* (23). The derivatives of the amino acids were identified and quantified by high pressure liquid chromatography using an Ultrasphere column (Altex) and a methanol gradient. Since cysteine, methionine, and tryptophan are degraded during the hydrolysis procedure, and proline does not form a derivative, these values were not determined. In addition, an accurate quantitation of glycine was not possible since the purified polypeptide, isolated by SDS-PAGE using glycine as a component of the elution buffer, contained trace amounts of this amino acid.

Results

The α-subunit of the AcCh receptor was isolated from receptor-enriched membrane fragments of the electric organ of *T. californica* and was digested with *S. aureus* V8 protease. The proteolytic fragments were resolved by SDS-PAGE and examined for their ability to bind ^{125}I-α-BgTx after electrophoretic transfer to nitrocellulose. At relatively low concentrations of V8 protease, several fragments of the α-subunit that were able to bind ^{125}I-α-BgTx were produced as evidenced by the single radioactive bands of apparent molecular weight 31,000 and 28,000 and the broad band in the 17,000–19,000 molecular weight range (Fig. 1A, *lane 2*). Increasing the V8 protease concentration generated relatively more of the lower molecular weight polypeptides and, at the highest enzyme concentration examined (α-subunit/V8, 5:1, w/w), only a single radioactive band of apparent molecular weight 17,000 was observed (Fig. 1A, *lane 4*). The polypeptide fragment(s) that was (were) associated with the lowest molecular weight region of ^{125}I-α-BgTx binding was (were) identified by staining a companion SDS-polyacrylamide gel with Coomassie blue. At low enzyme concentrations (Fig. 1B, *lane 2*) two major polypeptides of M_r = 19,000 and 17,000 were observed in the same molecular weight range as the ^{125}I-α-BgTx binding, suggesting that both fragments can bind the labeled toxin. At the highest enzyme concentration utilized there was a virtual loss of the 19-kDa polypeptide without an appreciable change in the amount of the 17-kDa polypeptide (Fig. 1B, *lane 4*). This resistance of the 17-kDa polypeptide fragment to further enzymatic degradation allowed us to generate this fragment in the absence of fragments of similar molecular weight and to isolate it by preparative SDS-PAGE in sufficient purity for further characterization. The purity and binding integrity of the isolated material were established by demonstrating that it migrated as a single band

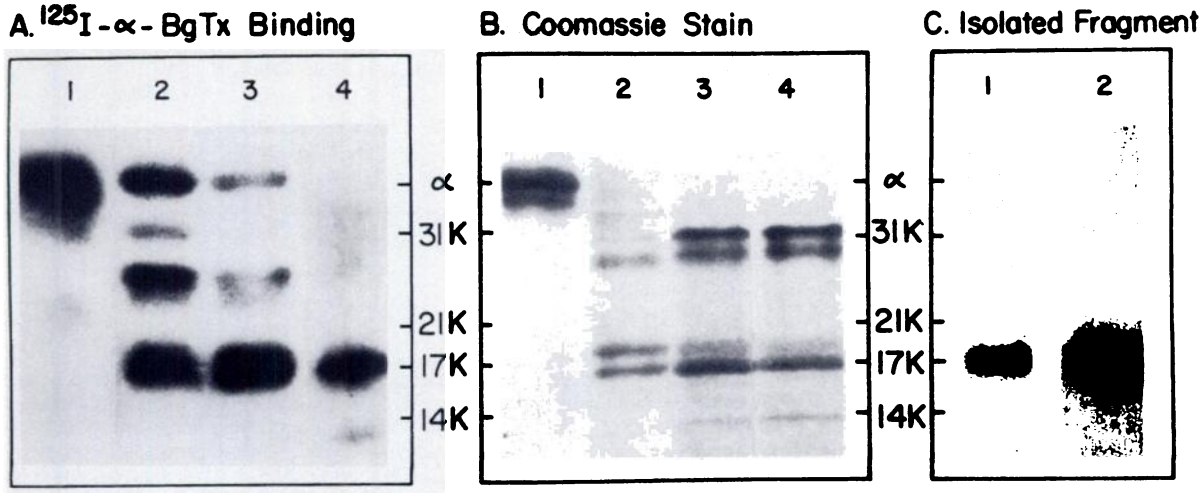

Fig. 1. A. Autoradiograph of ^{125}I-α-BgTx binding to proteolytic fragments of the α-subunit generated by V8 protease digestion. Ten μg of isolated α-subunit were digested with 0, 0.25, 0.75, and 2.0 μg of V8 protease (*lanes 1–4*). The proteolytic digestion products were separated on a 15% polyacrylamide gel, and the ability of the resolved polypeptide fragments to bind ^{125}I-α-BgTx was examined by the nitrocellulose protein transfer binding assay and autoradiography. B. Coomassie blue staining of the proteolytically derived fragments. In order to identify the polypeptide(s) in the region of toxin-binding activity, a companion gel to that utilized for transfer to nitrocellulose was stained with Coomassie blue. *Lanes 1–4* correspond to lanes 1–4 in A. The doublet band located around M_r = 31,000 in *lanes 3* and *4* is V8 protease. C. Coomassie blue staining and ^{125}I-α-BgTx binding to the isolated 17-kDa polypeptide fragment. The α-subunit (2.2 mg) was digested with V8 protease (0.4 mg). Following preparative SDS-PAGE a major polypeptide digestion product M_r = 17,000 was identified by the fluorescence detection technique (See Materials and Methods). Following electroelution, 160 μg of the 17-kDa polypeptide fragment were obtained. The purity of this polypeptide and its ability to bind ^{125}I-α-BgTx was assessed by resubmitting it to SDS-PAGE, staining with Coomassie blue (*lane 1*), and autoradiography after transfer to nitrocellulose and incubation with ^{125}I-α-BgTx (*lane 2*).

on an SDS-polyacrylamide gel and bound ^{125}I-α-BgTx following transfer to nitrocellulose (Fig. 1C, *lanes 1* and *2*, respectively). To locate the position of the 17-kDa polypeptide on the primary amino acid sequence of the α-subunit, the initial amino acid sequence at its amino terminus was determined by automated Edman degradation in a gas-phase sequencer. The amino acids identified as the major component (between 80 and 90% of the total amino acids released) on the first six cycles were Val, Asn, Gln, Ile, Val, and Glu, establishing a sequence that is identical to a unique sequence on the α-subunit beginning at Val 46. The V8 protease cleavage which generated the amino terminus of the 17-kDa polypeptide has therefore occurred between Glu 45 and Val 46 and is consistent with the known specificity of V8 protease for acidic amino acid residues (24). Based solely on considerations of the apparent molecular weight and the specificity of V8 protease for cleavage at glutamic and aspartic peptide bonds, the amino acid residue at the carboxy terminus is limited to Glu 129, Glu 241, or any one of the 11 intervening acidic amino acid residues (see Fig. 2). A partial amino acid composition of the isolated 17-kDa polypeptide fragment was also determined and the values obtained were fit by computer to those possible amino acid sequences beginning at Val 46. In Table 1, representative examples of these fits are presented. Polypeptide fragments extending to acidic amino acid residues between Glu 161, Glu 172, and Asp 180 were fit reasonably well by the data, whereas shorter or longer fragments were fit less well. Although fragments terminating in either Asp 152 or Asp 200 might be considered (based on the absolute sum of the differences and an experimental error of 10–20% for each amino acid determination) to be fit as well, both of these fragments are actually fit less well since each fragment exhibits a highly significant difference in one of the amino acid determinations: for Val 46-Asp 152, the expected value for Ser is 2 (observed 5.1) and for Val 46-Asp 200, the expected value for Tyr is 9 (observed 5.7).

The location of the carboxy terminus of the 17-kDa fragment was also restricted by the observation that this fragment stained positively with periodate/Schiff reagent.[1] Since Asn 141 is the only site of *N*-glycosylation on the entire primary sequence of the α-subunit (2), the 17-kDa fragment must extend at least to Asp 152. This conclusion is strengthened by our observation that, following treatment of the 17-kDa fragment by Endo F, an endoglycosidase that is specific for *N*-linked oligosaccharide chains, the mobility of the fragment on SDS-PAGE was increased and the deglycosylated polypeptide now migrated as a single band with an apparent molecular weight of 15,000 (Fig. 3). It is noteworthy that the 17-kDa fragment was readily deglycosylated by Endo F only following reduction by 2-mercaptoethanol and that, in its reduced state, the 17-kDa fragment did not bind ^{125}I-α-BgTx following transfer to nitrocellulose. Since the amino acid adjacent to Asn 141 is Cys 142, it appears that a disulfide linkage with this residue is involved in maintaining sufficient tertiary structure in the 17-kDa fragment to enable it to bind α-BgTx and also reduces the accessibility of Endo F to its cleavage site. Also shown in Fig. 3 are the results of Endo F treatment of the 19-kDa fragment which was isolated by preparative SDS-PAGE together with minor higher molecular weight fragments. This material, which bound ^{125}I-α-BgTx following transfer to nitrocellulose, did not stain postively for carbohydrate even when present in the gel at a relatively high concentration and following a 16-hr incubation with Schiff reagent. Since the mobility of this fragment on SDS-PAGE was not effected by Endo F treatment, the absence of carbohydrate on the 19-kDa polypeptide fragment was further substantiated.

[1] B. Oblas, R. H. Singer, and N. D. Boyd, unpublished observations.

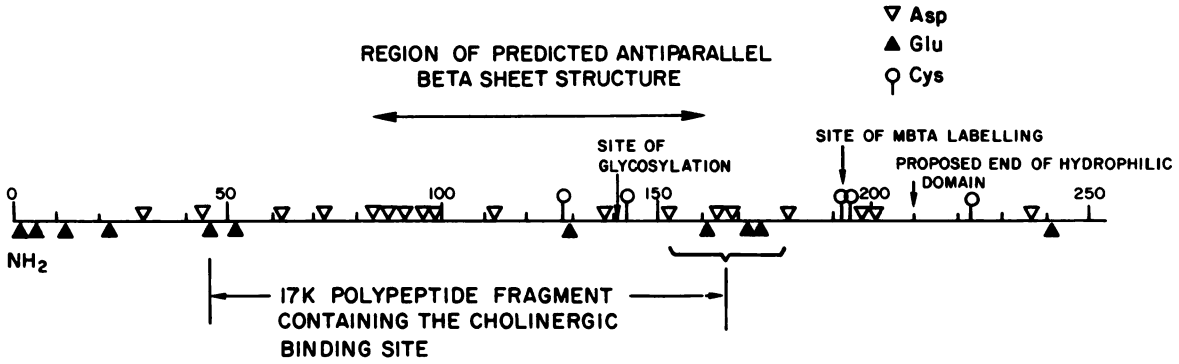

Fig. 2. Details of the primary structure of the amino terminal hydrophilic domain of the α-subunit. The location of the 17-kDa polypeptide fragment containing the cholinergic binding site was established by comparing the amino acid sequence at its amino terminus with the published sequence of the α-subunit. The most likely locations for the carboxy terminus are indicated and are based on considerations of the partial amino acid composition of the polypeptide fragment (see Table 1), the apparent molecular weight, M_r = 17,000, the enzyme specificity of V8 protease for acidic amino acid residues, and two identifying landmarks of this region: Asn 141, site of *N*-glycosylation, and Cys 192, site of MBTA labeling (see the text for details).

TABLE 1

Analysis of the partial amino acid composition of the 17-kDa polypeptide fragment

A partial amino acid composition of the 17-kDa polypeptide was obtained as described under Materials and Methods. For each of the polypeptide fragments beginning at Val 46 and ending at the indicated acidic amino acid residues, the experimentally determined relative amounts of the amino acid residues were analyzed by computer to obtain a constant multiplication factor that gave the optimum correspondence with the values calculated from the known sequence (indicated in parentheses). The reported values are the average of two separate determinations with a deviation between 10 and 20%.

Amino acid	Polypeptide sequence: Val 46 to						
	Glu 129	Asp 152	Glu 161	Glu 172	Asp 180	Asp 200	Glu 241
Ala	3.6 (4)	3.9 (4)	5.1 (4)	5.4 (4)	5.4 (4)	5.9 (4)	8.1 (4)
Arg	3.9 (5)	4.8 (5)	5.5 (5)	5.9 (6)	5.9 (6)	6.5 (7)	8.7 (8)
Asx[a]	10.4 (13)	12.8 (16)	14.5 (16)	15.6 (18)	15.6 (19)	17.1 (21)	23.1 (23)
Glx[b]	5.5 (5)	6.8 (7)	7.7 (8)	8.3 (9)	8.3 (10)	9.1 (10)	17.2 (13)
His	1.9 (1)	2.3 (2)	2.6 (2)	2.8 (2)	2.8 (2)	3.1 (3)	4.2 (4)
Ile	6.9 (7)	8.6 (10)	9.7 (11)	10.4 (11)	10.4 (11)	11.4 (11)	15.4 (16)
Leu	8.6 (11)	10.7 (12)	12.1 (12)	12.9 (13)	12.9 (13)	14.2 (14)	19.1 (20)
Lys	4.5 (3)	5.5 (4)	6.3 (5)	6.7 (5)	6.7 (6)	7.4 (7)	10.0 (7)
Phe	3.8 (2)	4.8 (4)	5.4 (4)	5.7 (5)	5.7 (5)	6.3 (5)	8.5 (10)
Ser	4.2 (2)	5.1 (2)	5.8 (4)	6.2 (6)	6.2 (7)	6.9 (7)	9.3 (9)
Thr	5.9 (4)	7.2 (7)	8.2 (8)	8.8 (9)	8.8 (9)	9.7 (11)	13.0 (14)
Tyr	3.5 (4)	4.2 (5)	4.8 (5)	5.2 (5)	5.2 (5)	5.7 (9)	7.7 (12)
Val	6.7 (7)	8.3 (8)	9.4 (9)	10.1 (9)	10.1 (10)	11.1 (11)	15.0 (15)
Absolute sum of differences	16.2	13.4	10.7	10.2	10.8	14.7	17.5
Molecular weight[c]	9,998	12,690	13,750	15,060	15,927	18,614	23,484

[a] Asp and Asn.
[b] Glu and Gln.
[c] The molecular weight values are calculated from the constituent amino acids and do not include the carbohydrate moiety, accounting for an additional 2–3 kDa, that is also present in the 17-kDa polypeptide fragment (see Fig. 3).

Another landmark on the primary sequence of the α-subunit which can aid in establishing the possible location of the carboxy terminus of the 17-kDa polypeptide fragment is Cys 192. This residue has been determined to be the primary site of covalent attachment by the cholinergic affinity ligand, MBTA (25). We therefore labeled the intact AcCh receptor with ^{3}H-MBTA and isolated the radiolabeled α-subunit which was then subjected to V8 protease digestion at a relatively low enzyme concentration (α-subunit/V8, 15:1, w/w). The digestion pattern was not apparently modified by prior MBTA labeling, and two polypeptide fragments of M_r = 17,000 and 19,000 were observed by Coomassie blue staining (Fig. 4A). Fluorography of the dried gel revealed that only the 19-kDa fragment that lacks carbohydrate and *not* the 17-kDa fragment that contains carbohydrate was radiolabeled (Fig. 4B). This result, which is in agreement with previous work by Gullick *et al.* (26), demonstrates that the 17-kDa polypeptide does not include Cys 192 and, thus, cannot extend beyond Asp 180 (Fig. 2).

Since Cys 192 is believed to be within 1 nm of the negative subsite that binds AcCh (27), and since this residue is not contained within the primary sequence of the 17-kDa polypeptide fragment, the possibility existed that the AcCh-and α-BgTx-binding sites were some distance apart on the primary sequence but were brought into close proximity by tertiary folding, thus accounting for the observed competitive interactions of these ligands with the intact α subunit (11). It was therefore conceivable that V8 protease could have cleaved between these two sites so that the 17-kDa polypeptide fragment could bind α-BgTx but might not contain the AcCh-binding site. A direct test of this possibility would involve examining the ability of AcCh to compete with α-BgTx binding to the 17-kDa polypeptide fragment. However, in view of the fact that AcCh bound to the isolated α-subunit with an apparently very weak affinity, with an IC_{50} value greater than 1 mM,[2]

[2] B. Oblas, R. H. Singer, and N. D. Boyd, unpublished observations.

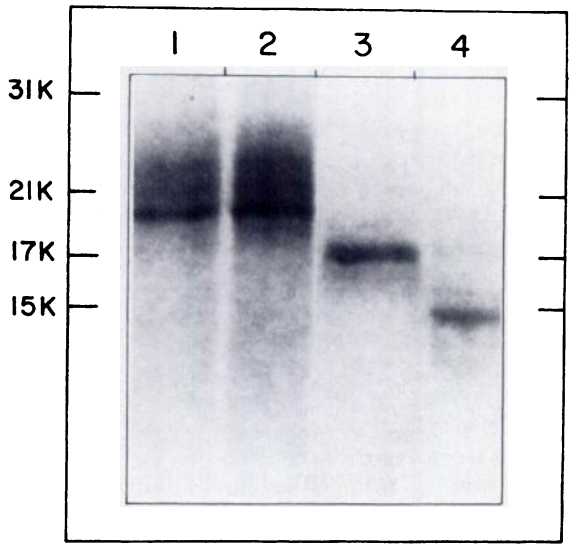

Fig. 3. The effect of Endo F on isolated 17- and 19-kDa polypeptide fragments of the α-subunit. Polypeptide fragments of M_r = 17,000 and 19,000 were generated by V8 digestion of the α-subunit and isolated by preparative SDS-PAGE. Following reduction by 2-mercaptoethanol in the presence of 0.1% SDS, the fragments were incubated for 4 hr at 37° in the presence or absence of Endo F. The incubation mixtures were then subjected to SDS-PAGE on a 15% gel followed by Coomassie blue staining. *Lanes 1* and *2:* the 19-kDa polypeptide fragment with and without Endo F treatment, respectively; *lanes 3* and *4:* the 17-kDa polypeptide fragment without and with Endo F treatment, respectively.

d-tubocurarine was utilized since it binds with a higher affinity to the intact α-subunit (11, 12) and competition studies on the native AcCh receptor have suggested that this ligand shares a common binding site with AcCh (28). As shown in Fig. 5, *d*-tubocurarine was able to inhibit virtually all of the radiolabeled toxin binding to the 17-kDa polypeptide indicating that the 17-kDa polypeptide fragment contains not only the α-BgTx-binding site but also the site of interaction of *d*-tubocurarine and, thus, presumably, the AcCh-binding site. Furthermore, the concentration at which *d*-tubocurarine displaced half the α-BgTx binding (IC_{50} = 0.4 mM) was not significantly different from that observed for the undigested α-subunit. Similarly, unlabeled α-BgTx was equipotent in displacing ^{125}I-α-BgTx binding to the 17-kDa polypeptide and to the undigested α subunit (IC_{50} = 100 nM).

Discussion

A toxin-binding polypeptide fragment of the α-subunit of the AcCh receptor was obtained by V8 digestion and preparative SDS-PAGE. The amino terminus of the fragment (M_r = 17,000) was determined by microsequencing and was unambiguously located at Val 46 on the primary amino acid sequence of the α-subunit. The carboxy terminus of the 17-kDa polypeptide fragment was less precisely determined to be between Asp 152 and Glu 180. This assignment was based on considerations of molecular weight, specificity of V8 protease, analysis of the partial amino acid composition, and the observation that the fragment contains Asn 141, the site of *N*-glycosylation, but not Cys 192, the site of alkylation by MBTA. Although the 17-kD fragment does not contain Cys 192, two cysteine residues, Cys 128 and Cys 142, are located within the proposed primary sequence. These residues appear to be linked by a disulfide bond for the following reasons. The isolation of both the α-subunit and its 17-kDa fragment were achieved by preparative SDS-PAGE without prior exposure to 2-mercaptoethanol since we have observed that disulfide bond reduction resulted in a substantial loss (approximately 80%) of ^{125}I-α-BgTx binding following transfer to nitrocellulose. If Cys 128 and Cys 142 were not linked to each other but formed disulfide bonds to any of the other cysteines present in the α-subunit, enzymatic cleavage at any one of the proposed glutamic or aspartic residues to generate the carboxy terminus of the 17-kDa polypeptide would necessarily yield two polypeptide fragments held together by disulfide linkages (see Fig. 2). However, we have observed that: 1) the 17-kDa polypeptide contains only a single amino terminus as revealed by microsequence analysis, and 2) reduction of the 17-kDa polypeptide by 2-mercaptoethanol does not alter the mobility of this polypeptide fragment on SDS-PAGE. It

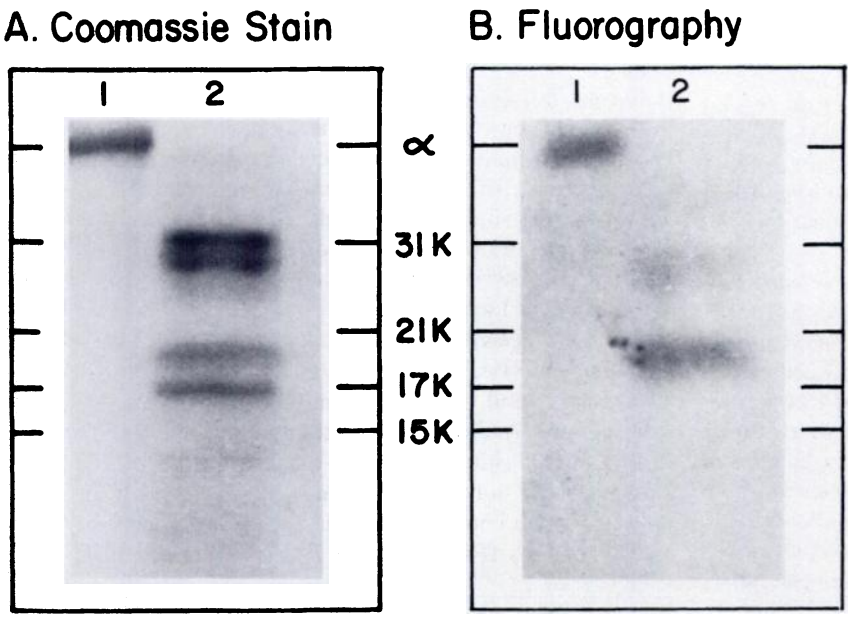

Fig. 4. V8 protease digestion of ^{3}H-MBTA-labeled α-subunit. The ^{3}H-MBTA-labeled α-subunit was isolated from the other receptor subunits by preparative SDS-PAGE and electroelution. A. Following incubation with V8 protease (α-subunit/V8 protease, 15:1, w/w) the resulting polypeptide fragments were resolved in a 15% polyacrylamide gel and stained with Coomassie blue. *Lane 1*, undigested radiolabeled α-subunit; *lane 2*, radiolabeled α-subunit following V8 digestion. The doublet band around M_r = 31,000 is V8 protease. B. In order to identify which of the polypeptide fragments were radiolabeled, the gel was prepared for fluorography (28). The dried gel was exposed to Kodak-X-Omat film for 5 weeks at −80° and the fluorogram was superimposed directly over the Coomassie blue-stained gel. The numbered lanes in B correspond to the lanes in A.

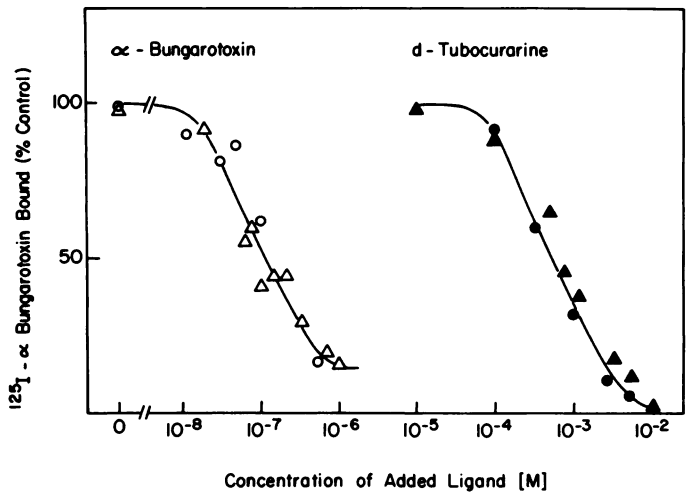

Fig. 5. ^{125}I-α-BgTx binding to the α-subunit and the 17-kDa polypeptide fragment in the presence of increasing concentrations of cholinergic ligands. The 17-kDa polypeptide was generated by digesting the α-subunit with V8 protease. Following SDS-PAGE and transfer to nitrocellulose, ^{125}I-α-BgTx binding to the 17-kDa polypeptide was determined quantitatively in the presence of increasing concentrations of nonradiolabeled α-BgTx (○) and *d*-tubocurarine (●). The binding of ^{125}I-α-BgTx to the undigested α-subunit was also determined in the presence of α-BgTx (△) and *d*-tubocurarine (▲) by the same technique. ^{125}I-α-BgTx binding to both the 17-kDa fragment and undigested α-subunit is expressed as a percentage of the binding in the absence of added cholinergic ligands.

therefore seems more likely that Cys 128 participates in a disulfide bond with Cys 142 and Cys 192 must form a disulfide bond with one of the four remaining cysteine residues: Cys 193, Cys 222, Cys 412, or Cys 420. Of these four residues, only Cys 193 has been proposed to be in a hydrophilic domain (2). Although a disulfide bond between adjacent cysteines has been proposed to be unlikely for steric reasons (29), an example of such a disulfide bridge has been reported recently for γ-transducin (30). It is also noteworthy that Cys 193 was the only cysteine residue other than Cys 192 that was reported to incorporate an appreciable amount of the ^{3}H-MBTA label (25). Recently, the binding of α-BgTx to AcCh receptors in which Cys 128, Cys 142, Cys 192, or Cys 193 of the α-subunit was converted to serine by site-directed mutagenesis of the cDNA was examined (31). Since AcCh receptor mutants with α 128 Ser or α 142 Ser showed virtually no α-BgTx binding, whereas AcCh receptor mutants with α 192 Ser or α 193 Ser showed nearly normal α-BgTx binding but a 10–30-fold increase in the IC_{50} for carbamylcholine, it was concluded that a disulfide bond exists between cysteine residues 128 and 142 as well as between cysteine residues 192 and 193, in agreement with the disposition of disulfide bonds proposed here.

In addition to the 17-kDa polypeptide fragment, we have identified a 19-kDa polypeptide fragment that also bound α-BgTx but, in contrast to the 17-kDa polypeptide fragment, was digested further by V8 protease at high enzyme concentration (see Fig. 1). Because of the susceptibility of the 19-kDa fragment to further digestion, we were unable to isolate it in sufficient quantity and purity for microsequencing and, thus, this fragment has been characterized less extensively. The isolated fragment, when subjected to SDS-PAGE, bound ^{125}I-α-BgTx following transfer to nitrocellulose but did not stain positively for carbohydrate. Moreover, the mobility of the fragment on SDS-PAGE was not altered following treatment by Endo F. Thus, this fragment cannot be simply a precursor of the 17-kDa polypeptide. Conti-Tronconi *et al.* (32) have reported the isolation of 17- and 19-kDa polypeptide fragments of the α-subunit that were also obtained by digestion with V8 protease. These fragments, which were both resistant to further digestion even at a high enzyme/substrate ratio, were found by microsequence analysis to share the same amino terminus beginning at Val 46. Since the 17-kDa polypeptide fragment was found to stain positively for carbohydrate whereas the 19-kDa polypeptide fragment stained less readily, these authors (32) have proposed that these fragments were derived from α-subunits that differed in their extent of glycosylation. Although the 17-kDa polypeptide that we have isolated and shown to bind α-BgTx is most likely identical to the 17-kDa fragment described by Conti-Tronconi *et al.* (32), the 19-kDa fragment obtained in this report by V8 digestion in a low concentration of SDS and without prior reduction by 2-mercaptoethanol seems to be different, since it did not appear to contain any carbohydrate and was not resistant to further digestion by V8 protease. This lack of carbohydrate, together with the observation that this fragment contains Cys 192, the site of alkylation by MBTA, suggests that the amino terminus of our 19-kDa fragment is located on the carboxyl side of Asn 141, the sole site of *N*-glycosylation on the α-subunit. Neumann *et al.* (33), using a similar experimental approach, have also identified a toxin-binding fragment (M_r = 18,000) that did not stain positively for carbohydrate and whose mobility on SDS-PAGE was not altered by treatment with endoglycosidase H and, thus, appears to correspond to the 19-kDa fragment described here. Moreover, since this fragment did not bind antibodies generated against a synthetic peptide corresponding to residues 126–143 of the α-subunit, these authors have also concluded that this fragment is located on the primary sequence of the α-subunit beyond Asn 141. In further agreement with our results are studies by Wilson *et al.* (34) in which a toxin-binding fragment of M_r = 19,000 was found not to bind ^{125}I-labeled conconavalin A.

If this consensus on the assignment of the position of the 19-kDa polypeptide fragment is correct, the location of the α-BgTx-binding site can be defined further since it is reasonable to assume that, if there is only one α-BgTx-binding site on the α-subunit, it must be contained within the segment of the primary sequence of the α-subunit that the 17- and 19-kDa polypeptide fragments have in common. Since the amino terminus of the 19-kDa polypeptide is predicted to be no closer to the amino terminus of the α-subunit than Asp 152 and the 17-kDa polypeptide cannot extend beyond residue Asp 180, the region of the α-subunit that binds α-BgTx would appear to be located between these residues, i.e., Asp 152–Asp 180. Recently, Boulter *et al.* (35) isolated a complementary DNA clone that was derived from RNA of the PC12 pheochromocytoma cell line and which encodes a protein homologous to the α-subunit of *Torpedo* electric organ and vertebrate muscle. Since the function of AcCh receptors in PC12 cells is not blocked by α-BgTx, a property shared with other AcCh receptors of neuronal origin, it might be expected that if the α-BgTx-binding site is located between residues 152 and 180, then the primary sequence of the α-subunit of the PC12 receptor would be less homologous in this region. In fact, the primary sequence proposed for the α-subunit of the PC12 receptor is poorly conserved in this region and, between residues 153 and 172, has only one

amino acid residue in common with the corresponding sequences in the α-subunit of vertebrate muscle and *Torpedo* electric tissue.

In addition to the 18-kDa polypeptide fragment that is apparently the same as our 19-kDa fragment, Neumann *et al.* (33) also observed a carbohydrate-containing 17-kDa polypeptide that bound antipeptide 126–143 but not antipeptide 1–20. On the basis of these results this fragment would seem to be similar to our 17-kDa polypeptide fragment. However, in marked contrast to our observation, this fragment did not bind α-BgTx following the transfer to nitrocellulose. Wilson *et al.* (34) have also identified a 17-kDa polypeptide fragment that bound ^{125}I-conconavalin A but did not appear to bind ^{125}I-α-BgTx. Since these results have led to a somewhat different conclusion by these two groups regarding the location of the α-BgTx binding region, it is useful to compare the experimental conditions that were employed in each study to generate the 17-kDa polypeptide fragments. In the experiments reported here, the α-subunit was isolated by preparative SDS-PAGE without prior reduction with 2-mercaptoethanol because, as discussed previously, this treatment resulted in a substantial loss in the ability of the reduced α-subunits to bind ^{125}I-α-BgTx following transfer to nitrocellulose. The α-subunits were then electroeluted from the excised gel slices in a low concentration of SDS (0.001%) and digested by V8 protease in the same low concentration of detergent. In contrast both Neumann *et al.* (33) and Wilson *et al.* (34) isolated the α-subunit following reduction by 3% 2-mercaptoethanol and generated their 17-kDa polypeptide fragment by treatment of the α-subunit with V8 protease in the presence of 0.1% SDS. Thus, it is possible that reduction by 2-mercaptoethanol and/or more extensive enzymatic cleavage under conditions of higher concentrations of the denaturing detergent may have resulted in the generation of a polypeptide fragment that, while apparently similar in molecular weight and primary sequence, is sufficiently different from our 17-kDa polypeptide fragment to have lost the ability to bind α-BgTx.

In summary, the isolation and biochemical characterization of a proteolytically derived fragment (M_r = 17,000) of the α-subunit that contains the cholinergic binding site have allowed us to locate the position of this fragment within the primary amino acid sequence of this subunit. This polypeptide fragment, rich in hydrophilic amino acids, is located in the middle of the larger of the two hydrophilic domains of the α-subunit, beginning at the amino terminus and extending to about amino acid residue 210. A consensus exists that this domain is located in its entirety on the extracellular side of the postsynaptic membrane (4, 7–9). An extracellular location for the 17-kDa polypeptide is supported by our observation that this polypeptide fragment contains carbohydrate. Computer analysis of the primary amino acid sequence of the α-subunit has revealed a unique region (approximately amino acid residues 80–160) which is predicted to be composed of a set of four β sheets closely packed in an anti-parallel orientation (2, 7). This sequence of highly ordered structure is only slightly smaller than the 17-kDa polypeptide fragment (see Fig. 2) and may account in part for the existence within the 17-kDa fragment of sufficient secondary and tertiary structure to bind α-BgTx and *d*-tubocurarine despite prior exposure to high concentrations of the denaturing detergent SDS. In addition, an ordered structure may be responsible for the observed resistance of the 17-kDa polypeptide to further enzymatic degradation, although it contains within its primary sequence multiple possible cleavage sites for V8 protease (Fig. 2).

The present study suggests that the AcCh-binding site lies in an extracellular region of the α-subunit and thus presumably at some distance from the transmembrane cation channel. The predicted rigid conformational structure of the cholinergic binding region may be significant in providing sufficient molecular movement in the receptor following the binding of AcCh to transduce spatially this binding into an increase in cation permeability.

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