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Binding of α-bungarotoxin to proteolytic fragments of the α subunit of *Torpedo* acetylcholine receptor analyzed by protein transfer on positively charged membrane filters

(electrophoretic transfer/maleimido-benzyltrimethylammonium/peptide map/protease/receptor–ligand interaction)

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ABSTRACT **Proteolytic fragments of the α subunit of the acetylcholine receptor retain the ability to bind α-bungarotoxin following resolution by polyacrylamide gel electrophoresis and immobilization on protein transfers. The α subunit of the acetylcholine receptor of *Torpedo* electric organ was digested with four proteases: *Staphylococcus aureus* V-8 protease, papain, bromelain, and proteinase K. The proteolytic fragments resolved on 15% polyacrylamide gels were electrophoretically transferred onto positively charged nylon membrane filters. When incubated with 0.3 nM ^{125}I-labeled α-bungarotoxin and autoradiographed, the transfers yielded patterns of labeled bands characteristic for each protease. The molecular masses of the fragments binding toxin ranged from 7 to 34 kDa, with major groupings in the 8-, 18-, and 28-kDa ranges. The apparent affinity of the fragments for α-bungarotoxin as determined from the IC_{50} value was 6.7×10^{-8} M. The labeling of fragments with α-bungarotoxin could be inhibited by prior affinity alkylation of receptor-containing membranes with 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide. These findings demonstrate that immobilized proteolytic fragments as small as 1/5 the size of the α subunit retain the structural characteristics necessary for binding α-bungarotoxin, although the toxin is bound to the fragments with lower affinity than to the native receptor. The effect of affinity ligand alkylation demonstrates that the α-bungarotoxin binding site detected on the proteolytic fragments is the same as the affinity-labeled acetylcholine binding site on the intact acetylcholine receptor.**

The nicotinic acetylcholine receptor (AcChoR) that functions to transduce a chemical signal into an electrical event in many neuronal systems has been well characterized both physiologically and biochemically (for review, see refs. 1 and 2). The AcChoR purified from the electroplaque of the electric ray *Torpedo californica* consists of four subunits present in the molar stoichiometry of $\alpha_2\beta\gamma\delta$ (3–5). This pentameric complex contains the agonist binding sites responsible for receptor activation and desensitization as well as the ion channel for cation flux through the membrane. Recent information on the primary amino acid sequences of the polypeptides comprising the receptor (5–9) provides a basis for analyzing and understanding these receptor functions at the molecular level.

One approach in elucidating receptor function is the dismantling of the intact receptor into its constituent parts and determination of the functions and properties of these simpler components. For example, the four subunits of the AcChoR have been isolated by gel electrophoresis in $NaDodSO_4$ and tested for α-bungarotoxin (α-BTX) binding after removal of the $NaDodSO_4$ (10). Saturable α-BTX binding could be demonstrated to the α subunit but not to the other chains. Further dissection of the receptor can be achieved through the preparation of peptide fragments by limited proteolysis of isolated subunits. This approach has been employed, for example, to compare the subunit structures of AcChoR from different sources (11, 12) and to characterize the domain specificity of anti-AcChoR monoclonal antibodies (13). One difficulty in utilizing parts of the AcChoR to study receptor function is that as the AcChoR is dismantled its characteristic functions may become less recognizable or no longer detectable. However, if small fragments of the AcChoR that retain essential functions can be identified, correlation of the structural characteristics responsible for these functions would be facilitated.

An important tool in the investigation of AcChoR has been the use of snake venom α-neurotoxins, such as α-BTX, which block the binding of cholinergic ligands to the receptor and thus are believed to bind at or near the AcCho binding site on the α subunit (14). These ligands that bind with a high degree of specificity and affinity can be used as probes to characterize the nature of receptor binding sites. Recently, we have introduced a novel approach in studying the molecular aspects of such receptor–ligand interactions (15). Protein blotting or electrophoretic transfer of dissociated proteins resolved by polyacrylamide gel electrophoresis onto immobilizing matrices has permitted the direct demonstration of various protein–ligand interactions, such as antibodies with antigens and lectins with glycoproteins (for review, see ref. 16). Protein blotting has been generally employed as a qualitative assay; however, we have refined this technique to a quantitative assay of protein–ligand association. In this manner, the binding of α-BTX to the isolated α subunit of the AcChoR of *Torpedo* was demonstrated directly and the kinetics and characteristics of the interaction were investigated (15). Furthermore, it could be demonstrated that the binding of α-BTX is specific and close to the physiologically relevant AcCho binding site on the receptor as it was inhibited by the cholinergic antagonist *d*-tubocurarine and the affinity alkylating agent 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide (MBTA).

In the present study, in an effort to gain further information on the nature of the AcCho binding site on the AcChoR, we have extended the protein transfer approach and analyzed α-BTX binding to isolated, immobilized peptide fragments prepared by proteolytic digestion of the α subunit of *T. californica* AcChoR. It is demonstrated that proteolytic fragments of the α subunit can be separated and immobilized

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Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; α-BTX, α-bungarotoxin; $LiDodSO_4$, lithium dodecyl sulfate; MBTA, 4-(*N*-maleimido)-α-benzyltrimethylammonium iodide; PCM, positively charged membrane.

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and that these fragments retain α-BTX-binding characteristics comparable to those of the intact α subunit. Preliminary reports of some of this work have appeared (17, 18).

MATERIALS AND METHODS

Preparation of *Torpedo* Electric Organ Membranes. Membranes were prepared from frozen electric organ tissue of *T. californica* (Pacific Biomarine, Venice, CA) as described (15). Based upon the number of α-BTX binding sites as determined by an immunoprecipitation assay (15), the AcChoR comprised about 5% of the total protein of this preparation.

Gel Electrophoresis and Proteolytic Digestion. *Torpedo* electric organ membrane was solubilized at room temperature for 1 hr in 50 mM Tris·HCl, pH 6.8/3% (vol/vol) 2-mercaptoethanol/2% (wt/vol) lithium dodecyl sulfate (LiDodSO$_4$; Sigma)/0.1% bromophenol blue. The sample was then resolved on a 10% polyacrylamide gel as described (19), except that EDTA was omitted from the upper reservoir buffer. Electrophoresis was carried out at 4°C at constant current (20 mA) overnight. Protein bands were visualized by placing the gel in 4 M sodium acetate as described (20). The band representing the α subunit of the AcChoR was excised from the gel and placed into a solution of 125 mM Tris·HCl, pH 6.8/0.1% (wt/vol) NaDodSO$_4$ for 30 min at room temperature. Gel slices were then placed into the wells of a NaDodSO$_4$/15% polyacrylamide gel and digested in the stacking gel with either *Staphylococcus aureus* V-8 protease (Miles Laboratories), papain (Sigma), bromelain (Sigma), or proteinase K (Merck) by the procedure of Cleveland *et al.* (21), except for the omission of EDTA from all solutions. Following electrophoresis, gels were immediately used for protein transfer.

Protein Transfer and Toxin Overlays. Electrophoretic transfer of the resolved proteolytic fragments from polyacrylamide gels onto positively charged membrane (PCM) filters (Zetabind; AMF/CUNO Specialty Materials Division, Meriden, CT) was performed as described by Gershoni and Palade (22). Filters were quenched in 1% (wt/vol) hemoglobin in 10 mM phosphate-buffered saline (pH 7.4, quenching buffer) at 45°C for 1 hr. Unless otherwise stated, filters were incubated in quenching buffer containing ≈0.3 nM ^{125}I-labeled α-BTX (New England Nuclear) for 8–15 hr at room temperature, washed four times for 5 min in cold (4°C) phosphate-buffered saline, and then autoradiographed with Kodak XAR-5 film and an intensifying screen (Dupont). Films were generally exposed for 24–72 hr. Quantitation of the amount of bound ^{125}I-labeled α-BTX was accomplished by excising from the filters the portion that contained the signal and an equivalent region without detectable signal to represent background binding. The excised portions were assayed for radioactivity in a Beckman Biogamma II counter. The net signal was calculated as the difference between the radioactivity of the signal-containing filter and the background-containing filter. Molecular weights of proteolytic fragments were determined by comparison with ^{125}I-labeled standards that were electrophoresed, transferred, and autoradiographed [*Escherichia coli* β-D-galactosidase, M_r 116,000; bovine serum albumin, M_r 67,000; bovine erythrocyte carbonic anhydrase, M_r 30,000; soybean trypsin inhibitor, M_r 20,100; bovine milk α-lactalbumin, M_r 14,400 (New England Nuclear); nerve growth factor, M_r 13,400; and α-BTX, M_r 8,000].

Affinity Alkylation with MBTA. The affinity alkylation of AcChoR with MBTA was performed essentially as described by Karlin (23). The MBTA was a gift from A. Karlin (Columbia University, New York). AcChoRs in *Torpedo* electric organ membranes were reacted with MBTA as described (15). The α subunit derived from MBTA-treated AcChoR was resolved on 10% polyacrylamide gels and digested with papain, *S. aureus* V-8 protease, or bromelain. The fragments were separated by gel electrophoresis, transferred, and incubated with ^{125}I-labeled α-BTX, and the amount of toxin bound to fragments and intact α subunit was determined by autoradiography and quantitation of bands as described above.

RESULTS

^{125}I-labeled α-BTX was found to bind on protein blots to several peptide fragments generated by proteolysis of the α subunit of *Torpedo* AcChoR. Digestion of the α subunit excised from LiDodSO$_4$/10% polyacrylamide gels with various proteases generated peptide fragments that were resolved on NaDodSO$_4$/15% polyacrylamide gels and subsequently electroeluted onto PCM filters. After transfer, the filters were processed and incubated with ^{125}I-labeled α-BTX. In the case of untreated α subunit, after autoradiography a single radioactive band at an electrophoretic mobility corresponding to ≈40 kDa was detected (Fig. 1, lane A). After digestion by *S. aureus* V-8 protease (Fig. 1, lane B), papain (Fig. 1, lane C), bromelain (Fig. 1, lane D), and proteinase K (Fig. 1, lane E), a pattern of bands was obtained that was consistent and highly reproducible for each protease. Digestion with *S. aureus* V-8 protease resulted in three areas that bound ^{125}I-labeled α-BTX: single bands at mobilities corresponding to ≈32 kDa and ≈27 kDa and a closely spaced, major group of bands (triplet) at 18–19 kDa. With extensive digestion, a band at 9 kDa was observed. Proteolysis with papain produced a more complex pattern with bands at mobilities of ≈33–34, 28, 25, 16–17, 10, and 7 kDa. Bromelain treatment resulted in fragments of ≈28, 19, and 15–16 kDa. Proteinase K treatment produced bands at 29, 21, and 8 kDa. The 9-kDa

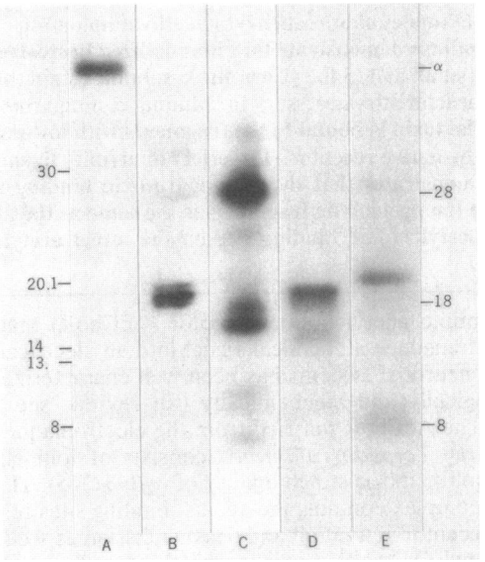

FIG. 1. ^{125}I-labeled α-BTX binding to protein transfers of α subunit and peptide fragments of α subunit. The α subunit of *Torpedo* AcChoR from electric organ membranes was visualized in LiDodSO$_4$/10% polyacrylamide gels in 4 M sodium acetate and excised. Gel slices containing ≈8 μg of α subunit were then placed into the wells of NaDodSO$_4$/15% polyacrylamide gels and digested for 30 min in the stacking gel with proteases. Lanes: A, no protease; B, 1.0 μg of *S. aureus* V-8 protease; C, 1.0 μg of papain; D, 1.0 μg of bromelain; and E, 2.0 μg of proteinase K. Following electrophoresis, the resolved polypeptides were transferred to Zetabind filters and incubated with ≈0.3 nM ^{125}I-labeled α-BTX for 8–15 hr at room temperature. After four 5-min washes in cold (4°C) phosphate-buffered saline, the filters were autoradiographed for 24–72 hr. The positions of molecular mass standards (in kilodaltons) are shown on the left. The position of the α subunit and the molecular masses of some of the fragments are indicated on the right.

fragment produced by V-8 protease, the 8-kDa fragment produced by proteinase K, and the 7- and 10-kDa fragments resulting from papain treatment were the smallest fragments found to bind ^{125}I-labeled α-BTX after transfer to PCM filters with this technique. There were few peptides of the same electrophoretic mobility produced by the different proteases; however, there appeared to be major groupings of fragments binding ^{125}I-labeled α-BTX around 28, 18, and 8 kDa. Silver staining of gels revealed that the proteases generated a number of fragments that did not bind α-BTX (data not shown).

The α subunit was incubated with increasing amounts of protease to determine the smallest fragments that could be detected. As expected, lower molecular mass fragments binding α-BTX became more prominent at the expense of higher molecular mass fragments when the ratio of protease to α subunit was increased (Fig. 2). However, above a certain ratio of protease to α subunit (e.g., 2.5 μg of papain, Fig. 2), the characteristic pattern of labeled bands did not change appreciably, even when the amount of protease was increased 10-fold.

The amounts of ^{125}I-labeled α-BTX binding to the intact α subunit and the proteolytic fragments on filters were compared. The numbers of counts present on strips of filters containing the α subunit and equal-sized filters containing proteolytic fragments derived from the same amount of α-subunit were measured. It was found that the same amount of toxin was bound to the α subunit and to the proteolytic fragments (Table 1).

To further characterize the observed toxin binding to proteolytic fragments and gain information on the affinity of the toxin for the fragments, competition experiments with unlabeled α-BTX were performed. Fragments generated by *S. aureus* V-8 protease were separated by gel electrophoresis and then transferred onto PCM filters. Competition of ^{125}I-labeled α-BTX binding was accomplished by including in the incubation solution increasing amounts of unlabeled α-BTX. Following autoradiography of the filter, the group of bands at ≈18 kDa was excised and the amount of ^{125}I-labeled α-BTX bound was quantitated. The results of such an experiment are presented in Fig. 3, which shows that the amount of ^{125}I-labeled α-BTX bound to the fragments decreased as the amount of unlabeled α-BTX increased. The concentration of unlabeled α-BTX that produced 50% inhibition of ^{125}I-labeled α-BTX binding to the fragments on the filters was 6.7 × 10^{-8} M. Although the competition at the other bands was not quantitated, the autoradiograph revealed that labeled α-BTX binding to all of the fragments observed was comparably reduced by increasing amounts of unlabeled toxin.

To determine whether the binding of α-BTX to the immobilized proteolytic fragments is to a physiologically significant AcCho binding site, the effect of affinity alkylation with MBTA on toxin binding was tested. MBTA has been used to correlate the binding of toxin to intact receptor with that of isolated α subunit (15). A similar approach has been adopted here to correlate the binding of toxin to isolated α subunit with binding to the various proteolytic fragments. Samples of *Torpedo* membrane were affinity alkylated with increasing amounts (0–10 μM) of MBTA. The resolved α subunit was digested with proteases and binding of labeled α-BTX to the α subunit and the proteolytic fragments was compared.

Table 1. ^{125}I-labeled α-BTX binding to α subunit of the AcChoR and proteolytic fragments

Enzyme	Radioactivity, cpm	
	α subunit	Fragments
V-8 protease	9,775	10,624
Papain	12,962	13,227
Bromelain	6,055	5,981
Proteinase K	14,850	15,186

After incubation in ^{125}I-labeled α-BTX, strips of filter containing undigested α subunit and equal-sized adjacent strips containing proteolytic fragments derived from digestion of the same amount of α subunit were excised and assayed for radioactivity in a gamma counter. For each protease, the cpm obtained with a single filter containing the α subunit and a filter containing proteolytic fragments are shown. The cpm obtained represent the total of toxin binding to α subunit or fragments and background binding to the filter.

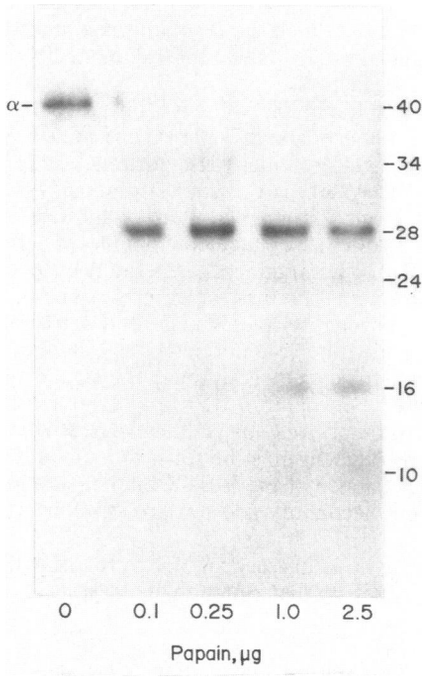

FIG. 2. ^{125}I-labeled α-BTX binding to protein transfer of α subunit digested with increasing amounts of papain. The α subunit was excised from a $LiDodSO_4$/10% polyacrylamide gel of *Torpedo* electric organ membrane and gel slices containing ≈8 μg of α subunit were placed into the wells of a $NaDodSO_4$/15% polyacrylamide gel. The indicated amounts of papain were then added to the respective wells and digestion was allowed to occur in the stacking gel. Following electrophoresis, the gel was transferred to a Zetabind filter, which was incubated with ≈0.3 nM ^{125}I-labeled α-BTX. After four 5-min washes in cold (4°C) phosphate-buffered saline, the filter was autoradiographed for 70 hr. The position of the α subunit and the molecular masses (in kDa) of the proteolytic fragments are indicated.

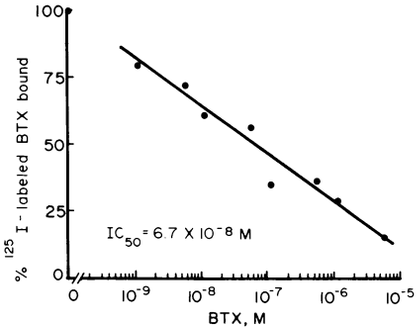

FIG. 3. Competition of ^{125}I-labeled α-BTX binding with unlabeled α-BTX. Identical protein transfers of 8 μg of α subunit digested with 2 μg of *S. aureus* V-8 protease were incubated for 3 hr with 0.2 nM ^{125}I-labeled α-BTX and variable amounts of unlabeled α-BTX. The transfers were washed in cold (4°C) phosphate-buffered saline and autoradiographed. The radioactivity associated with the group of bands at 18–19 kDa was determined by excising the bands from the filter, assaying them in a gamma counter, and subtracting counts from an equivalent area of background.

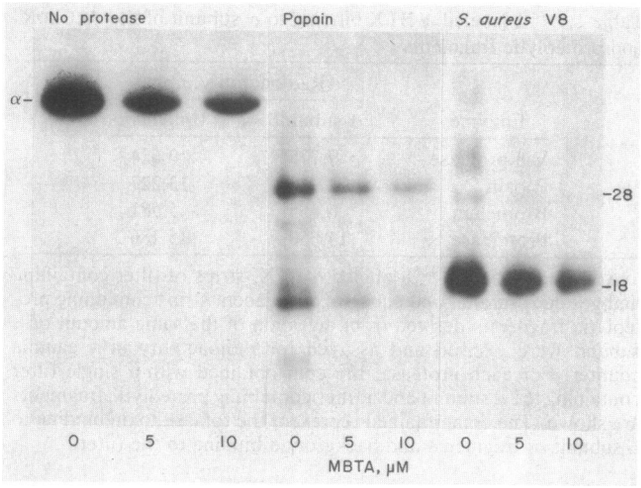

FIG. 4. Effect of MBTA treatment on the binding of ^{125}I-labeled α-BTX to protein transfers of α subunit and proteolytic fragments. The indicated MBTA concentrations (0, 5, and 10 μM) were used to alkylate *Torpedo* electric organ membrane (≈0.3 μM AcChoR). The α subunits from the respectively labeled membranes were isolated from $LiDodSO_4$/10% polyacrylamide gels, placed into the wells of $NaDodSO_4$/15% polyacrylamide gels, and either treated with no protease or digested in the stacking gel with 1 μg of papain or *S. aureus* V-8 protease. The gels were transferred to Zetabind filters and the filters were incubated with 0.3 nM ^{125}I-labeled α-BTX for 8 hr. After washing with cold (4°C) phosphate-buffered saline, the filters were autoradiographed for 24 hr. The positions of the α subunit and of the 28-kDa (papain) and 18-kDa (V-8 protease) fragments used for quantitation are indicated.

The effect of prior affinity alkylation of the α subunit with MBTA on the binding of labeled α-BTX to the intact α subunit and to proteolytic fragments generated by *S. aureus* V-8 protease or papain treatment is illustrated in a composite autoradiograph (Fig. 4). Prior MBTA treatment of the membranes did not change the peptide patterns obtained after digestion with the proteases (see Fig. 1). More important, however, is that all of the fragments as well as the intact α subunit showed reduction in the binding of ^{125}I-labeled α-BTX as the amount of MBTA was increased. Similar results were obtained with fragments generated by bromelain treatment.

Quantitation of this apparent reduction in binding was achieved by excising the α subunit, the 28-kDa fragment produced by papain treatment, and the ≈18-kDa group produced by *S. aureus* V-8 protease and by measuring the amount of ^{125}I-labeled toxin bound. In this manner, the effect of MBTA treatment on toxin binding to the α subunit could be compared to the effects on the proteolytic fragments. MBTA alkylation inhibited toxin binding to the α subunit and to both of the proteolytic fragments to the same degree at every concentration of MBTA tested (Fig. 5). The concentration of MBTA that caused 50% inhibition of toxin binding was ≈5 μM.

DISCUSSION

The major finding of this study is that proteolytic fragments of the α subunit of the AcChoR as small as 7 kDa retain the ability to bind ^{125}I-labeled α-BTX following resolution by $NaDodSO_4$/gel electrophoresis and immobilization on PCM filters. The apparent affinity of the fragments for α-BTX as determined from the IC_{50} value was 6.7×10^{-8} M. This value agrees closely with the binding affinity of 10^{-7} M for the immobilized, intact α subunit (15). Similarly, α-BTX inhibited labeled α-BTX binding to the α subunit isolated by gel electrophoresis in $NaDodSO_4$ after removal of the $NaDodSO_4$ with an IC_{50} of 2×10^{-7} M (10) and to isolated α subunit immobilized on nitrocellulose with an IC_{50} of about 10^{-7} M (24). For both isolated α subunit and proteolytic fragments, the binding affinity is about 10^4 times lower when compared to that of the native Triton X-100-extracted AcChoR. Recently, it has been reported that partial recovery of high-affinity binding can be achieved in the presence of 0.02% $NaDodSO_4$ (25).

Digestion of α subunit of *Torpedo* receptor yielded characteristic cleavage patterns for each protease used. The patterns obtained with *S. aureus* V-8 protease and papain were similar to those obtained by other investigators (25–27). In the case of all of the proteases used in this study, the patterns were stable and did not change beyond a certain range of protease concentration. Such stability in light of the relatively broad substrate specificity of three of these proteases (papain, bromelain, and proteinase K) indicates that secondary and tertiary structural factors must play a role in determining the sites at which the α subunit is attacked. In addition, treatment in 0.1% $NaDodSO_4$ does not abolish these conformational factors.

The smallest fragments we have observed that bind α-BTX are fragments of about 7–10 kDa obtained with V-8 protease, papain, and proteinase K digestion. An apparent advantage of our system is the ability to detect binding of α-BTX to these small fragments. Tzartos and Changeux (25), using a DEAE-filter assay, detected binding of α-BTX to soluble 34- and 27-kDa fragments generated by papain treatment, in agreement with the results reported here. They also reported the production of 17- and 10-kDa fragments, but, unlike our results, they did not detect any binding of α-BTX to these smaller fragments. Although the smallest fragments we have observed to bind α-BTX are about 1/5 the size of the intact α subunit, it is not yet determined what the minimal size of a fragment must be for it to include the primary amino acid sequence of the α-BTX binding site and to possess sufficient secondary and tertiary structure for binding to occur.

The affinity alkylating reagent MBTA reacts with a sulfhydryl group formed by the reduction of a cystine at or near the

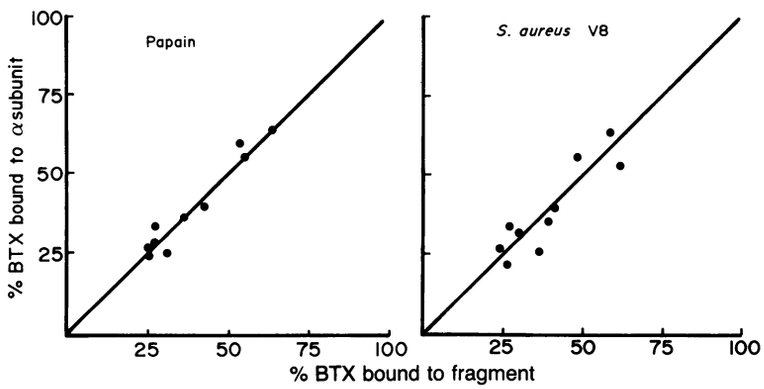

FIG. 5. Quantitation of the effect of MBTA treatment on ^{125}I-labeled α-BTX binding. Samples of *Torpedo* membrane were treated with increasing concentrations of MBTA (0–10 μM). Following electrophoresis, the variously labeled α subunits without digestion or after treatment with papain or *S. aureus* V-8 protease were transferred and incubated with 0.3 nM ^{125}I-labeled α-BTX. The amount of labeled toxin associated with α subunit treated with no protease, the 28-kDa fragment from papain digestion, and the ≈18-kDa group from *S. aureus* V-8 protease was determined by measurement of radioactivity of bands as described for the concentrations of MBTA used. The amount of toxin bound by the uninhibited samples (without MBTA) was taken as 100%.

AcCho binding site (2). In addition, α-neurotoxin blocks binding of MBTA to the receptor and MBTA reduces the binding of toxin, indicating that the toxin binding site is near the AcCho binding site as well (28). Gullick *et al.* (27) described labeled fragments derived from treatment of [^{3}H]MBTA-labeled α subunit with V-8 protease and papain. We have found that these as well as other fragments bind labeled α-BTX. It is possible, however, that the MBTA and α-BTX sites are some distance apart in the primary amino acid sequence but are brought into close proximity by tertiary structure. Thus, the possibility exists of separating the two sites on different proteolytic fragments if cleavage should occur between the MBTA and α-BTX binding sites. The approach employed here of studying the binding of α-BTX to fragments immobilized on filters allows fragments that are affinity alkylated with MBTA to be tested for α-BTX binding. It was demonstrated that alkylation of the intact α subunit with MBTA resulted in decreased binding of α-BTX to all fragments that bound α-BTX generated by V-8 protease and papain (Fig. 4) as well as by bromelain (data not shown). In no fragment were we able to separate the two binding sites—that is, we did not detect a fragment for which treatment with MBTA did not result in a reduction in the binding of ^{125}I-labeled α-BTX.

Quantitation of the reductions in ^{125}I-labeled α-BTX binding to intact α subunit and to proteolytic fragments on PCM filters after affinity alkylation with MBTA demonstrated that binding was inhibited to the same extent in both cases. Previously, it was determined that prior MBTA alkylation inhibited the binding of α-BTX to isolated α subunit on PCM filters and to intact, detergent-solubilized receptor to the same degree (15). Together, these findings show that the α-BTX binding site detected on the proteolytic fragments is the same as the physiologically relevant, high-affinity site on the intact AcChoR. However, because the isolated α subunit and proteolytic fragments exhibit only low-affinity binding, it appears that the high-affinity α-BTX binding site is converted to a low-affinity binding site upon dissociation, denaturation, and immobilization.

Both MBTA and the related alkylating agent bromoacetylcholine have been widely used to investigate the site on the AcChoR responsible for receptor activation. A number of studies have suggested that these agents react preferentially with one of the two α-BTX binding sites per monomeric AcChoR (1, 29). Under saturating conditions of alkylation, however, both toxin binding sites appear to be labeled (29–31). In our hands, using an immunoprecipitation assay to quantitate toxin binding to detergent-solubilized *Torpedo* AcChoR, we find, consistent with other reports, that 5 μM MBTA leads to a 50% loss of toxin binding sites. At higher concentrations, a near complete blockade of toxin binding is observed. Although differential reactivity of various agents towards the two α subunits may occur in the intact receptor due to the different microenvironments of the subunits, such nonequivalence of reactivity would not be necessarily expected in the case of the isolated α subunit immobilized on a nylon support.

PCM filters as an immobilizing matrix have proven to be useful for the identification of proteolytic fragments of the α subunit of the AcChoR that bind α-BTX. Because of the high capacity of these filters (22) and their apparent ability to bind relatively small fragments, it should be possible to extend this approach to the screening of additional, smaller fragments generated by sequential protease treatments. Use of proteases with more specific substrate specificities or under varied reaction conditions could permit dissection of the structural features of the α-BTX binding site in a rigorous fashion similar to restriction analysis of nucleic acids. In addition, other probes (e.g., lectins, monoclonal antibodies) can be used to characterize further the fragments that retain the ability to bind α-BTX. It should be possible to obtain sufficient chemical amounts of the fragments that bind α-BTX for further characterization, including amino acid sequence analysis. Because the deduced primary amino acid sequence of the α subunit as well as the potential site of N-linked glycosylation are known (6), limited NH_2-terminal sequence analysis of fragments identified by this technique should make it possible to identify the location of these fragments in the primary sequence and thereby more clearly define the region of the α subunit involved in the binding of AcCho.

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