Segment α182–198 of *Torpedo californica* acetylcholine receptor contains a second toxin-binding region and binds anti-receptor antibodies

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The area around Cys-192 and Cys-193 is thought to be a functionally important part of the α-subunit of the acetylcholine receptor. We have synthesized peptide α182–198 of the α-chain of the *Torpedo californica* acetylcholine receptor and investigated the binding to the peptide of α-bungarotoxin, cobratoxin and antibodies raised against acetylcholine receptor. The results showed that the synthetic peptide α182–198 contains a second toxin-binding region and also binds a considerable fraction of anti-receptor antibodies. We also report here the toxin-binding activity of synthetic peptide α125–148 of the human acetylcholine receptor which has been previously localized as a toxin-binding region in the α-chain of the *Torpedo* receptor.

Acetylcholine receptor *Toxin-binding region* *Synthetic peptide* *Cobratoxin* *α-Bungarotoxin* *Antigenic site* *Antibody*

1. INTRODUCTION

The nicotine acetylcholine receptor $(AChR)^+$ from *Torpedo* electroplaque is a multi-subunit glycoprotein that mediates ion flux across the cell membrane in response to binding of acetylcholine [1–4]. The receptor complex is composed of 4 different subunits existing in a stoichiometry $\alpha_2\beta\gamma\delta$. The primary structures of all 4 subunits have been elucidated from cDNA sequences [5–7]. Several studies have shown that the α-subunit contains the acetylcholine-binding site [8–10]. The regulatory activity of this site(s) is inhibited by binding to an α-neurotoxin [e.g. α-bungarotoxin (BgTX) or cobratoxin (CbTX)] from snake venom [11] and, therefore, these toxins serve as very specific, high-affinity probes for the acetylcholine-binding site [12].

Based upon sequence analysis, structural topology and site-directed mutagenesis, it has been proposed that the area around the invariant cysteine residues 128 and 142, and/or cysteine residues 192–193 is/are essential for binding to acetylcholine and toxin to receptor [5–7,14]. More direct evidence was recently obtained [13] by showing that a synthetic peptide corresponding to residues 125–147, with a disulfide bond between Cys-128 and Cys-142, of the *Torpedo* receptor possessed high binding activity to both ^{125}I-labelled BgTX and [^{3}H]acetylcholine.

This paper reports the synthesis and binding activity of a peptide corresponding to residues 182–198 of the *Torpedo* α-subunit, to BgTX and CbTX as well as to anti-AChR antibodies. The toxin-binding activity of this peptide is compared to the *Torpedo* loop peptide αT125–147 [13] and to the human loop peptide α125–148 corresponding to residue 125–148 of the human α-subunit, also synthesized here for the first time. These studies have shown that the synthetic peptide αT182–198 possessed high binding activity to ^{125}I-labelled BgTX and CbTX. This region also bound considerable amounts of anti-AChR antibodies.

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2. MATERIALS AND METHODS

2.1. *Isolation of* Torpedo *AChR receptor*

The preparation of AChR from the electric organ tissue of *Torpedo californica* (Pacific Bio-Marine Laboratories) was carried out as described by Froehner and Rafto [15]. Receptor was solubilized from membranes in buffer containing 1% Triton X-100 and then purified by affinity chromatography on CbTx (*Naja naja siameensis*)-Sepharose CL4B. Binding activity was determined by DE81 filter assay [16]. Purity and subunit composition of AChR were checked by polyacrylamide gel electrophoresis in SDS [17].

2.2. *Synthesis and purification of the peptides*

The peptides αT182–198 and αH125–148 (fig.1) were synthesized by the Merrifield solid-phase method [18]. The side chain protecting groups used on the N^{α}-*t*-butyloxy amino acids (Vega Biochemicals) were: γ- and β-benzyl esters for glutamic and aspartic acids, guan-NO_2 for arginine, *O*-benzyl for serine and threonine, 2-chloro-Cbz for lysine ϵ-NH_2, 2,6-dichlorobenzyl for tyrosine hydroxyl, 4-methylbenzyl for cysteine SH and dinitrophenyl for the imidazole group of histidine. The 1-hydroxybenzotriazole/DCC coupling procedure [19] was used to incorporate the Asn, Gln, Arg, and His residue, while the coupling of all other amino acids was performed by symmetric anhydrides [20]. A 6 molar excess of the *t*-Boc-amino acid and 3 equivalents of DCC were used with respect to the starting load on the resin. The *t*-Boc group was removed with 40% trifluoroacetic acid in CH_2Cl_2 (v/v). The dinitrophenyl protecting group on histidine was removed from the completed peptide by thiolysis with benzenethiol. Cleavage of the peptide from the resin was performed by anhydrous HF containing anisole (30 min, −20°C).

The peptides were purified by gel filtration on Sephadex G-25 (Pharmacia) in 0.05 M triethylamine to obtain the monomeric fractions [13] followed by ion-exchange chromatography on DEAE-Sephacel [13]. Homogeneity of the products was monitored by high-voltage paper electrophoresis [21]. Amino acid analysis of acid hydrolysates was performed on a Beckman 6300 amino acid analyzer. For the determination of tryptophan αT182–198, the peptide was hydrolysed with 3 M *p*-toluenesulfonic acid [22]. Cysteine was determined on the analyzer as cysteic acid after oxidation of the hydrolysates with performic acid.

2.3. *Adsorbent-binding studies*

Proteins (BgTX, CbTX, immune IgG) and peptides were labelled with ^{125}I (Amersham) using the chloramine-T method [23]. Protein-linked ^{125}I was assayed by precipitation with 10% (w/v) trichloroacetic acid. Mouse antibodies against TAChR elicited by immunization of mice (Swiss Webster) in complete Freund's adjuvant at 3 weekly intervals. The antisera used here were 80 day bleed from mice. The IgG fractions of anti-AChR antisera were prepared as described [26].

Coupling of the peptides to CNBr-activated Sepharose CL-4B [24] was carried out under the optimum conditions [25]. Adsorbents of BgTX and CbTX were prepared by the same method. Adsorbents of unrelated proteins and unrelated synthetic peptides [25,26] were used as controls for non-specific binding. AChR was coupled in a similar manner to that used for other proteins, except that the coupling buffer was 0.2 M $NaHCO_3$, pH 9.5, containing 0.1 M NaCl and 0.2% Triton X-100. The binding of either ^{125}I-labelled BgTX or CbTX to AChR to peptide adsorbents or, con-

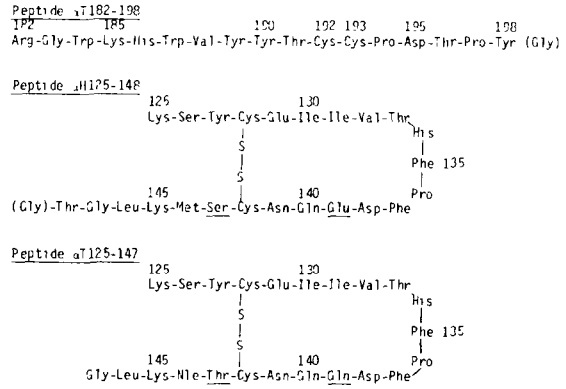

Fig.1. Covalent structures of the synthetic peptides studied here. Peptides αT182–198 and αH125–148, which were synthesized in the present work, were both started on glycine-resin for ease of synthesis. Therefore, in these two peptides, the C-terminal glycine is not part of the natural sequence in *Torpedo* or human AChR. The synthesis and activity of peptide αT125–147 have been reported [13].

versely, of labelled peptides to BgTX or CbTX adsorbents was determined by a quantitative solid-phase radiometric binding assay [25,27].

The solid-phase radiometric binding assay was also used for studying antibody binding activity of the peptide [25]. Peptide-Sepharose conjugates were incubated with ^{125}I-labelled immune IgG from mouse anti-AChR antisera and antibody binding was determined as in [25]. Adsorbents of unrelated proteins and peptides were as controls. Also, the binding to AChR peptide of antibodies against unrelated proteins served as additional controls. Antibody binding was determined by a double-antibody assay [28] using ^{125}I-labelled rabbit and mouse IgG to monitor the binding of unlabelled mouse anti-AChR antibodies.

In all binding experiments, non-specific binding was determined by titrating, under identical conditions, equivalent volumes of uncoupled Sepharose and Sepharose coupled to unrelated proteins and synthetic peptides.

3. RESULTS

3.1. *Purification and characterization of the synthetic peptides*

After purification, the synthetic peptides were homogeneous by peptide mapping. In amino acid analysis, the synthetic peptides had compositions which were in agreement with those expected from their sequence (fig.1). Their amino acid compositions (with the values expected from the sequence in parentheses) were: peptide αT182–198, Asp, 1.09 (1); Thr, 1.92 (2); Pro, 2.06 (2); Gly, 0.98 (1); Val, 0.95 (1); Tyr, 2.85 (3); His, 0.85 (1); Lys, 0.88 (1); Arg, 0.94 (1); Trp, 1.98 (2); 1/2 Cys (determined as cysteic acid), 2.05 (2); peptide αH125–148, Asp, 2.07 (2); Thr, 1.90 (2); Ser, 1.80 (2); Glu, 3.02 (3); Pro, 0.96 (1); Gly, 1.05 (1); Val, 0.98 (1); Met, 0.86 (1); Ile, 1.92 (2); Leu, 1.02 (1); Tyr, 1.00 (1); Phe, 2.13 (2); His, 1.01 (1); Lys, 2.06 (2); 1/2 Cys (determined as cysteic acid), 1.95 (2). The synthesis, characterization and activity of peptide αT125–147 have been reported [13].

Peptide αT182–198 was confirmed to be a monomer by gel filtration on a calibrated column of Sephadex G-25 in 0.05 M triethylamine with an apparent M_r of 2000. Reaction of the peptide with 5,5′-dithiobis(2-nitrobenzoic acid) in 5 M guanidine HCl at pH 8.0 as described by Habeeb [29] revealed the presence of two free thiol groups. This was confirmed by reaction of the peptide with a 50 molar excess of ICH_2CONH_2. After removal of excess reagent by gel filtration on Sephadex G-15, amino acid analysis of acid hydrolysates of the derivative gave 1.9 mol *S*-carboxymethylcysteine/mol peptide. Thus, the thiol groups on the vicinal cysteine residues 192 and 193 were free and did not form a disulfide bond. On the other hand, the monomeric peptide αH125–148 did not react with the reagents unless it was first reduced by $NaBH_4$. After reduction, peptide αH125–148 showed the presence of 2.0 thiol groups, clearly indicating that the monomer exists as a loop with a disulfide bond between Cys-128 and Cys-142 (fig.1). It should also be noted that the peptide αT125–147 is a monomer with a disulfide bond between Cys-128 and Cys-142 [13].

3.2. *Binding of ^{125}I-labelled peptides to toxin adsorbents*

Both synthetic regions, 125–148 and 182–198, were bound to toxin adsorbents (fig.2). However,

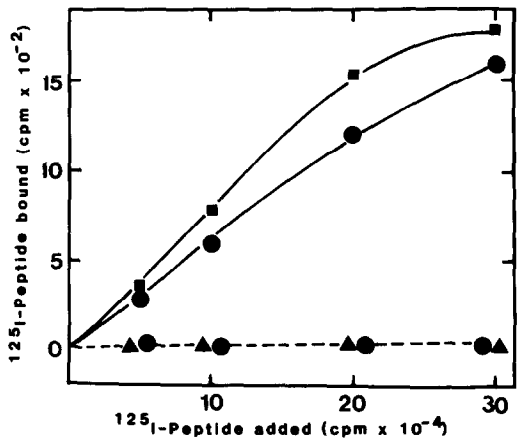

Fig.2. Binding of ^{125}I-labelled peptides αT182–198 and αH125–148 to CbTX adsorbent. A fixed volume (15 μl of a 1:1 suspension) of CbTX adsorbent was titrated with different amounts of radioiodinated peptides (5×10^4, 10^5, 2×10^5 and 3×10^5 cpm). The specific activities of the labelled peptides were: peptide αT182–198, 1.63 μCi/nmol; peptide αH125–148, 5.45 μCi/nmol. (——) Binding to CbTX of: (●) peptide αT182–198; (■) peptide αH125–148. (– – –) Binding of the two peptides to: (▲) adsorbent of bovine serum albumin and, (●) adsorbents of human hemoglobin synthetic peptide α45–56 [26].

under these conditions ^{125}I-labelled peptides αT125–147 or αH125–148 showed lower binding activity than ^{125}I-labelled αT182–198. Specificity of this binding was confirmed by the finding that unrelated ^{125}I-labelled proteins and peptides did not bind to toxin adsorbents. In general labelled peptides bound very poorly to toxin (fig.2) because, in all likelihood, several amino acid side chains (tryptophan, tyrosine, histidine, methionine, cysteine and cystine), some of which may be essential contact residues, can potentially undergo oxidation by chloramine-T [30] during radioiodination resulting in derivatives with very poor binding activity. More definitive results were obtained by binding labelled toxins onto peptide adsorbents, therefore, this method was used.

3.3. *Binding of ^{125}I-labelled toxins to peptide adsorbents*

The binding of BgTX to adsorbents of the synthetic peptides was first determined by titrating a fixed amount of ^{125}I-labelled BgTX with varying amounts of peptide adsorbents. The binding values in the plateau region are summarized in table 1 and show that both peptides bound amounts of BgTX that were considerable, being 40–74% relative to the amount bound by AChR: non-specific binding to unrelated proteins and peptides was only 3–5% relative to AChR.

Table 1

Binding of BgTX to synthetic peptides αH125–148 and αT182–198

Sepharose adsorbent	^{125}I-labelled BgTX bound (cpm)
AChR	36800 ± 889
Peptide αT192–148	27278 ± 1217
Peptide αH125–148	14670 ± 256
Bovine serum albumin	1334 ± 66
Hemoglobin A	1595 ± 10
Myoglobin	1848 ± 101
Hemoglobin peptide α45–56[a]	1493 ± 59
'Nonsense' peptide[b]	1361 ± 77

[a] This represents an adsorbent, used here as a control, of a synthetic peptide corresponding to residues 45–56 of the α-chain of human hemoglobin A. The synthesis and characterization of this peptide have been reported [26]

[b] 'Nonsense' peptide is a synthetic peptide that does not correspond to any part of AChR, and its adsorbent is used here as a control. The synthesis and characterization of this peptide have been reported [25]

Binding to protein and peptide adsorbents was measured by a quantitative radiometric binding assay, using a fixed amount of ^{125}I-labelled BgTX (10^5 cpm) and increasing amounts of adsorbents. The results below summarize the mean plateau binding values of triplicate experiments

Binding studies were also carried out on the absorbents of AChR and of the two peptides αT182–198 and αH125–128 synthesized here as well as the adsorbent of the previously reported [13] peptide of *Torpedo* αT125–147, using a fixed amount of adsorbent suspension and increasing amounts of ^{125}I-labelled CbTX and BgTX. In titration with CbTX (fig.3), the binding curves of the

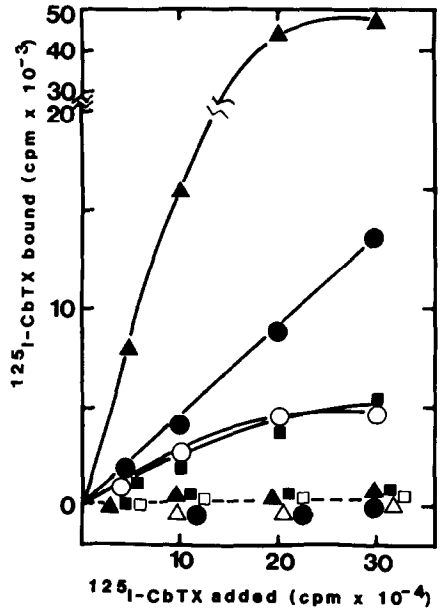

Fig.3. Binding of ^{125}I-labelled CbTX to receptor, synthetic peptides and controls. Increasing amounts of labelled CbTX were added to a fixed volume (25 μl of 1:1 suspension) of each adsorbent. (——) Titration curves of the toxin with adsorbents of (▲) AChR, (●) peptide αT182–198, (■) peptide αH125–148, (○) peptide αT125–147. (– – –) Binding of the toxin to control adsorbents of (▲) bovine serum albumin, (■) sperm whale myoglobin, (●) human hemoglobin synthetic peptide α45–56, (△) human adult hemoglobin and (□) nonsense peptide [25]. The specific activity of the labelled toxin was 18.95 μCi/nmol.

human and *Torpedo* peptides αH125–148 and αT125–147 (representing equivalent structural regions on the α-chain of the respective species) were identical. On the other hand, the binding curve of the *Torpedo* peptide αT182–198 was considerably higher. The binding curve of AChR under the same condition is shown in fig.3.

Similar titration studies were carried out with a fixed amount of each adsorbent and increasing amounts of ^{125}I-labelled BgTX (fig.4). In titration with BgTX, AChR showed, as expected, the highest binding. The highest peptide binding activity was exhibited by peptide αT182–198 followed by peptides αH125–148 and αT125–147, in that order. Peptide αT182–198 showed, on the adsorbent, apparent affinities with BgTX and CbTX of 6.6×10^{-8} and 1×10^{-7} M, respectively, while the corresponding values for peptide αH125–148 were 1.5×10^{-7} and 2.2×10^{-7} M. Labelled toxins did not bind to adsorbents of unrelated proteins and peptides, thus confirming the specificity of the previously discussed binding results.

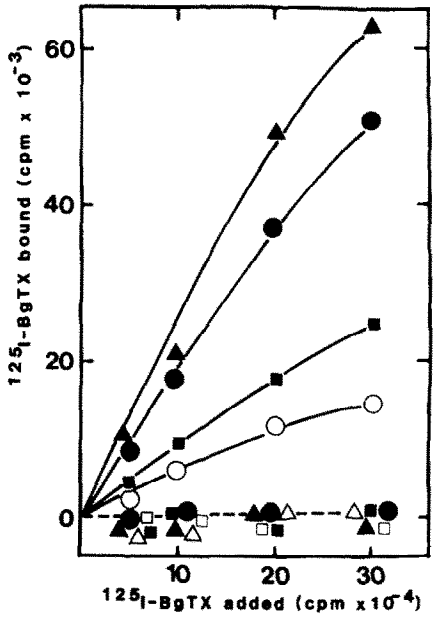

Fig.4. Binding curves of ^{125}I-labelled BgTX to AChR, synthetic peptides and controls. Fixed volumes of adsorbents (25 μl of 1:1 suspension) were titrated with increasing amounts of ^{125}I-labelled BgTX. (——) Titration curves of the toxin with adsorbents of (△) AChR, (●) peptide αT182–198, (■) αH125–148, (○) αT125–147. (– – –) Binding behavior to control adsorbents of (▲) bovine serum albumin, (■) sperm whale myoglobin, (●) human hemoglobin synthetic peptide α45–56, (△) human adult hemoglobin and (□) nonsense peptide. The specific activity of the labelled toxin was 18.2 μCi/nmol.

3.4. *Antibody-binding studies*

Quantitative radioimmunoadsorbent titrations were carried out both with ^{125}I-labelled anti-AChR antibodies and by a double-antibody assay. In a given titration, a fixed amount of antibody and increasing amounts of adsorbents were used. The results in table 2 summarize the mean plateau binding values by both assays and show that peptide αT182–198 binds a considerable fraction of anti-AChR antibodies. The antibody binding to unrelated control proteins and peptides is negligible (table 2).

Table 2

Binding of anti-AChR antibody to synthetic peptide αT182–198 of the AChR α-chain of *Torpedo californica* and to unrelated controls

Sepharose adsorbent	Antibody bound (cpm) by:	
	(A) Direct antibody	(B) Double-antibody
AChR	79707 ± 1270	125125 ± 2240
αT182–198	17261 ± 1011	28007 ± 1120
Bovine serum albumin	236 ± 18	118 ± 27
Myoglobin	98 ± 20	368 ± 72
Myoglobin peptide 1–6	−214 ± 45	−4 ± 10
Nonsense peptide	−144 ± 32	373 ± 50

(A) Results of the direct antibody assay in which ^{125}I-labelled mouse anti-AChR antibody (2.5×10^5 cpm) was incubated (4°C, 18 h) with varying amounts of peptide or protein adsorbents; (B) results obtained by the double-antibody assay. An aliquot (20 μl) of unlabelled mouse anti-AChR antisera (prediluted 1:500, v/v) is incubated (4°C, 18 h) with increasing amounts of adsorbent. This is followed, after washing, with an aliquot (2.5×10^5 cpm) of ^{125}I-labelled rabbit anti-mouse IgG. After washing (5 times) all the tubes on the centrifuge, they were counted on a gamma counter. All dilutions, solvents and washing solutions were 1% fetal bovine serum in PBS. The results, which summarize plateau binding values (obtained with 100 μl of 1:1 suspension of adsorbents), have been corrected for non-specific binding [(A) 1693 cpm; (B) 1570 cpm] by an equal volume of uncoupled Sepharose

4. DISCUSSION

Previously, it was reported [13] that a synthetic peptide corresponding to sequence 125–147 of the α-subunit of the *Torpedo* AChR had high binding activity toward BgTX. We report here that the 17-residue synthetic peptide corresponding to the sequence 182–198 of the α-chain of the *Torpedo* AChR (αT182–198) has very high binding activity toward both CbTX and BgTX.

As a result of site-directed mutagenesis experiments [14], the residues Cys-192 and Cys-193 of the α-subunit have been proposed to play a role in receptor binding. However, since it is difficult to separate the effect of replacing a residue from that of any attending conformational readjustments which result from that replacement, it remains usually uncertain whether the effect of a substitution on the binding activity of a protein directly implicates the residue that has been altered as a 'contact' residue or whether it may simply be the by-product of conformational changes.

The results presented here provide direct evidence that the receptor has a second toxin-binding region which resides within the α-chain fragment 182–198. Peptide 125–148 of the α-chain of the human AChR was synthesized in order to confirm further the universal activity of this binding region in two different species. In this region of the α-chain, the human sequence differs from the *Torpedo* sequence at positions 139 and 143. The residues Gln-139 and Thr-143 in *Torpedo* α-chain are replaced in the human protein by glutamic acid and serine, respectively. The substitution of threonine by serine is conservative and probably will not adversely affect the binding activity of this region. On the other hand, substitution of glutamine by glutamic acid will result in a drastic charge alteration which would more likely be disruptive to the binding activity if this is an essential contact residue. Also, it should be noted that in the synthetic peptide αT125–147, Met-144 was replaced by norleucine [13].

The present results show that the human synthetic peptide αH125–148 possesses considerable binding activity with both BgTX and CbTX, which is comparable to that of the *Torpedo* synthetic peptide αT125–147. However, in the binding to BgTX, the human peptide shows higher affinities. Thus, it may be concluded that residue 139 on the peptides has little or no involvement in the binding of the toxin to AChR. But this replacement and/or the replacements at position 143 and 144 may modulate the region's affinity for the toxin.

The results reported here also show that this second toxin-binding region, residues α182–198, is also recognized as a major antigenic site by antibodies raised against native AChR. A considerable fraction of anti-AChR antibodies is bound specifically by this region of the α chain. Whether antibodies against this region play a role in the autoimmune disease, myasthenia gravis, is now under investigation. It is not implied that this antigenic site includes all of the residues 182–198, but rather it resides within these residues.

Thus, it has been shown unequivocally that the α-chain of AChR has a second toxin-binding region which has been localized to fall within, but may not necessarily include all of the residue 182–198. This region may not be independent of the region within residue 121–148. It is not possible to distinguish at present whether these two regions represent distinct binding sites on the α-chain for toxin or whether they are two 'faces' within a single binding site, the binding energy of each face being sufficient to form a complex with one face of a toxin-binding site. The results also do not exclude the possibility that regions and/or residues from other parts of the α-chain may contribute to the toxin-binding site(s). Determination of the three-dimensional structure of AChR or its α-chain should help to clarify this question.

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