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Localization and synthesis of the acetylcholine-binding site in the α-chain of the *Torpedo californica* acetylcholine receptor

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The sequence of the α-chain of the acetylcholine receptor of *T. californica* has been determined by recent cloning studies. The integrity of the disulphide bond between Cys-128 and Cys-142 has been shown to be important for the maintenance of the binding activity of the receptor, thus implicating the regions around the disulphide bridge in binding with acetylcholine. In the present work, a synthetic peptide containing this loop region (residues 125–147) was synthesized. Solid-phase radiometric binding assays demonstrated a high binding of ^{125}I-labelled α-bungarotoxin to the synthetic peptide. It was further shown that the free peptide bound well to [^{3}H]acetylcholine. Additonal experiments demonstrated that pretreatment of peptide 125–147 with 2-mercaptoethanol destroyed its binding activity, clearly showing that the integrity of the disulphide structure was essential for binding. Unlabelled acetylcholine also inhibited the binding of labelled acetylcholine to the synthetic peptide. The region 125–147, therefore, contains essential elements of the acetylcholine binding site of the *Torpedo* receptor.

The nicotinic acetylcholine receptor (AChR) has been the subject of intensive research in recent years because of its central role in post-synaptic neuromuscular transmission (Reynolds & Karlin, 1978; Karlin, 1980; Changeux, 1981), and its involvement as a major auto-antigen in the human disease myasthaenia gravis (Lennon & Lambert, 1981; Tzartos *et al.*, 1982; Gomez & Richman, 1983). Several studies have shown that the α-subunit (M_r 40000) of the receptor from *T. californica* or *T. marmorata* (Sobel *et al.*, 1977; Moore & Raftery, 1979; Tzartos & Changeux, 1983) contains the acetylcholine binding site which regulates cation fluxes of the ionophore channel in the subsynaptic membrane. The regulatory activity of this site has also been shown to be inhibited by the binding of α-neurotoxins (e.g. α-BTX or cobratoxin) from snake venom (reviewed in Lee, 1979).

Recently, cDNA clones of the genes encoding the structure of the subunits of AChR of *T. californica* have been sequenced (Noda *et al.*, 1982, 1983*a*; Claudio *et al.*, 1983). Based upon sequence analysis and structural topology of the α-subunit, it has been proposed that the invariant cysteine residues 128 and 142 comprise the disulphide bridge, the integrity of which is essential for the binding of acetylcholine to the receptor (Karlin, 1980; Noda *et al.*, 1982, 1983*b*, 1983*c*; Devillers-Thiery *et al.*, 1983).

We have undertaken studies to localize by peptide synthesis the acetylcholine binding site in both human and *Torpedo* receptors. In the present work, a synthetic peptide correponding to residues 125–147 of the *Torpedo* receptor was synthesized. Studies showed that the synthetic peptide possessed a high binding activity to both ^{125}I-labelled α-BTX and [^{3}H]acetylcholine. These results establish that the α-chain region 125–147 contains essential elements of the acetylcholine binding site of the receptor of *T. californica*.

Abbreviations used: AChR, native acetylcholine receptor of *T. californica*; α-BTX, α-bungarotoxin; PBS, 0.01M-sodium phosphate, pH7.2, containing 0.15M-NaCl.

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Experimental

AChR was prepared from the electroplax organ tissue of *T. californica* according to the procedures of Gershoni *et al.* (1983) and Froehner & Rafto (1979).

α-BTX (25 μg) was radiolabelled with ^{125}I

(1.0mCi) by the chloramine-T method (Hunter & Greenwood, 1962) in siliconized tubes (Sigmacote; Sigma Chemical Co.) and free label was removed by gel filtration on a Sephadex G-25 column (Pharmacia) eluted with PBS containing 0.2% bovine serum albumin. Specific radioactivity of the labelled preparations was routinely 12.5–16.0 μCi/μg of protein.

The peptide 125–147 was synthesized with the *t*-butoxycarbonyl derivatives of L-amino acids (Vega Biochemicals) on Merrifield (1963) resin esterified to *t*-butoxycarbonylglycine (0.77 mmol of glycine/g of resin) according to the established procedures of this laboratory (Koketsu & Atassi, 1973, 1974). Completion of coupling after each residue was monitored by the ninhydrin method of Kaiser *et al.* (1970). The amino acid sequence of the synthetic peptide is given in Fig. 1. In the synthetic peptide Met-144 was replaced by norleucine. The side chain protecting groups employed in synthesis were: ε-2-chlorocarbobenzyloxy for lysine; *O*-2,6-dichlorobenzyl for tyrosine; γ- and β-benzyl esters for glutamic and aspartic acid, respectively; *N-imidazole*-tosyl for histidine; *O*-benzyl for serine and threonine; *S*-4-methylbenzyl for cysteine; hydroxybenzotriazole was employed in the coupling of histidine, glutamine and asparagine.

After synthesis, the peptide was cleaved from the resin support by treatment with 7ml of anhydrous HF containing 0.5ml of anisole for 30min at -20°C. The crude peptide was then dissolved in trifluoroacetic acid containing 1.0% 2-mercaptoethanol, precipitated with cold diethyl ether three times, and lyophilized. The peptide (155mg) was initially purified on a column (2.5cm × 50cm) of DEAE-Sephacel (0.05M-pyridine/acetate, pH4.8) followed by ascending chromatography on a column (2.5cm × 55cm) of Sephadex G-25 in 0.025M-acetic acid, pH3.5. The Sephadex column was precalibrated with dinitrophenyl-alanine, the synthetic peptides of sperm-whale myoglobin (peptide 137–153; Young & Atassi, 1983) and human haemoglobin A (α-chain peptide 45–56; Kazim & Atassi, 1980) and Blue Dextran. The appropriate fractions containing peptide monomers were pooled, neutralized to pH7.0 with 1.0M-pyridine, and lyophilized. Reaction of the monomeric peptide with 5,5′-dithiobis-(2-nitrobenzoic acid) indicated the absence of free thiol groups. Determination of the disulphide bonds by using the method described by Habeeb (1972), which involves reduction with $NaBH_4$ followed by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid), confirmed the presence of one disulphide bond (two thiol groups after reduction). The yield of monomeric pure peptide was 25.1 mg. Analysis by high-voltage paper electrophoresis was in pyridine/acetate (pH3.65). Amino acid analysis was performed on hydrolysates (in triplicate; 0.1mg of peptide, 22h, 110°C, constant-boiling HCl) on a Beckman 6300 amino acid analyser.

The proteins AChR, bovine serum albumin and haemoglobin, and peptide 125–147, were coupled to CNBr-activated Sepharose CL-4B (March *et al.*, 1974) under the optimum conditions determined by Twining & Atassi (1979). The AChR protein was coupled in a manner similar to albumin and haemoglobin, except that 0.2% Triton X-100 was added to the coupling buffer. Adsorbents of the control peptides haemoglobin β-chain 1–15, myoglobin 56–62 and myoglobin 137–153 were also prepared. The amounts of proteins and peptides coupled in adsorbents were determined by amino acid analysis of acid hydrolysates of aliquots of each adsorbent. Protein adsorbents contained 0.9–1.2mg/ml, while peptide adsorbents had 0.5–0.7mg/ml of packed volume.

The binding of ^{125}I-labelled α-BTX to AChR and peptide 125–147 was determined by a quantitative solid-phase radiometric binding assay as described in detail by Twining & Atassi (1979) and Kazim & Atassi (1980) with the following modifications. Sepharose adsorbents (AChR, peptide 125–147 and control proteins and peptides) were incubated in varying amounts with labelled α-BTX (328000c.p.m.) in 200 μl of PBS containing 0.4% sodium cholate and 0.02% sodium dodecyl sulphate for 18h at 24°C (Tzartos & Changeux, 1983). Adsorbents were then washed five times with PBS containing 0.1% sodium dodecyl sulphate and 0.5% Triton X-100, and then counted for the presence of bound toxin in a Beckman 4000 gamma counter. In

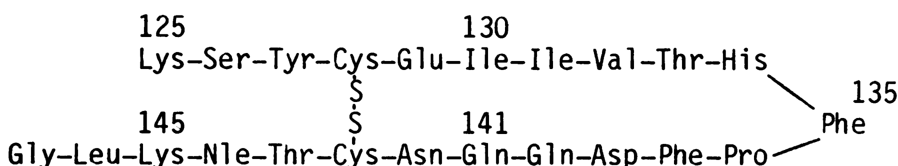

Fig. 1. *Amino acid sequence of AChR peptide 125–147 synthesized from the reported structure of the α-chain (Noda et al., 1982, 1983c) of the acetylcholine receptor of T. californica*
In the synthetic peptide Met-144 was substituted by norleucine (Nle).

a similar experiment, the binding of [^{3}H]acetylcholine (3.7Ci/mmol; Amersham International) to peptide 125–147 was determined by incubation of 100 μl volumes of absorbents with labelled acetylcholine (5.9×10^{6} c.p.m.) in PBS containing 0.2% albumin. After 18h, the absorbents were washed five times with PBS containing 0.05% sodium dodecyl sulphate, dried on filter paper, and counted for bound radioactivity in a Beckman LS 2800 liquid-scintillation counter. The non-specific binding in both experiments was determined by titrating equal volumes of Sepharose CL-4B and control adsorbents in parallel binding experiments.

In separate experiments, the binding of [^{3}H]-acetylcholine to free, unconjugated peptide 125–147 was measured by the following solution-phase binding assay. Briefly, an aliquot (1.5, 2.5, 5, 10 or 100 μg; 0.51–34 nmol) of the synthetic peptide was incubated in 200 μl of PBS with 2.5 μCi of [^{3}H]acetylcholine for 3h at 24°C. The peptide was then separated from unbound label by gel filtration on a column (1.1 cm × 36 cm) of Sephadex G-25 eluted with PBS. Fractions (0.6 ml) were collected and aliquots (50 μl) were measured for the presence of [^{3}H]acetylcholine in a Beckman LS 2800 scintillation counter. In similar experiments, the peptide was preincubated with 2-mercaptoethanol or unlabelled acetylcholine (100 mmol excess) before adding labelled acetylcholine. In a parallel binding experiment, 1.0 mg of a control peptide (haemoglobin A α-chain 45–60) was incubated with labelled acetylcholine under identical conditions. The elution volume of the haemoglobin peptide 45–60 was measured by its A_{230}, and the radioactivity of [^{3}H]acetylcholine was measured in 50 μl aliquots by liquid scintillation.

Finally, binding of [^{3}H]acetylcholine to a free peptide (2.5 μg) was also examined under equilibrium condition (Hummel & Dreyer, 1962) by gel filtration on a column (0.5 cm × 37 cm) of Sephadex G-25 which was equilibrated and eluted with PBS containing 3×10^{6} c.p.m. of [^{3}H]acetylcholine/ml. Fractions (0.3 ml) were collected and aliquots (50 μl) of these were counted by liquid scintillation.

Results and discussion

The synthetic peptide comprising the amino acid sequence 125–147 (see Fig. 1) of the α-chain of the *Torpedo* receptor was synthesized and purified to homogeneity. In the α-chain sequence, methionine occupies position 144 and it was replaced by norleucine in the synthetic peptide to avoid problems caused by oxidation of the thioether side chain of methionine which might occur during isolation, purification and disulphide bond formation. The purified monomer was homogeneous by high voltage paper electrophoresis in heavily loaded conditions. The monomeric peptide did not react with the reagent 5,5′-dithiobis-(2-nitrobenzoic acid) unless it was first reduced by treatment with $NaBH_4$. This indicated that cysteine residues 128 and 142 were covalently bonded as a disulphide bridge. In amino acid analysis, the synthetic peptide monomer gave a composition which was in excellent agreement with that expected from its sequence. The amino acid composition was: Asp, 2.2 (2); Thr, 2.17 (2); Ser, 1.07 (1); Glu, 3.19 (3); Pro, 1.03 (1); Gly, 1.06 (1); ½Cys, 2.02 (2); Val, 1.10 (1); Nle, 1.20 (1); Ile, 2.07 (2); Leu, 1.06 (1); Trp, 0.91 (1); Phe, 2.28 (2); His, 0.99 (1); Lys, 2.23 (2). The cysteine (Cys) residues were determined as cysteic acid.

The binding of peptide 125–147 to ^{125}I-labelled α-BTX is given in Table 1, which summarizes the plateau binding values of each absorbent tested. As demonstrated in these experiments, the synthetic peptide possessed a high binding activity to labelled toxin (19090 c.p.m.) relative to the binding of toxin by the receptor (51130 c.p.m.). The binding of α-BTX to unrelated control proteins and peptides was negligible (see Table 1). To test whether the α-BTX binding activity

Table 1. *Binding of α-BTX to synthetic peptide 125–147*

Binding to protein and peptide absorbents was measured by quantitative radiometric binding assays. The results below summarize the mean plateau binding values of four replicate tubes incubated with toxin in two separate experiments. The total amount of labelled α-BTX added to each tube was 328000 c.p.m. All values have been corrected for non-specific binding of labelled toxin to CL-Sepharose 4B (2547 c.p.m.) at plateau binding levels.

Sepharose absorbent	^{125}I-labelled α-BTX bound (c.p.m.)
Native AChR	51130 ± 1150
Peptide 125–147	19090 ± 670
Bovine serum albumin control	1200 ± 220
Haemoglobin A	1830 ± 330
Haemoglobin peptide 1–15	1210 ± 180
Myoglobin peptide 56–62	860 ± 110
Myoglobin peptide 137–153	1580 ± 270

exhibited by peptide 125–147 correlated with the physiologically relevant acetylcholine binding site, the direct binding of this peptide to acetylcholine was examined. Adsorbents of AChR and peptide 125–147 demonstrated a moderate binding activity to [^{3}H]acetylcholine (see Table 2). The binding of labelled acetylcholine to control adsorbents, however, was negligible.

The appreciably lower binding activity of peptide 125–147 to labelled acetylcholine was suspected to be due to some loss of peptide binding efficiency as a result of covalent coupling to Sepharose supports. Immobilized peptides have been previously reported to suffer loss in binding activity because they might assume rigid conformations that are not favourable for binding (Twining & Atassi, 1978), or because of the attachment of essential contact amino acid side chains which contribute chemically to the binding energy of the region (Atassi *et al.*, 1979; Sakata & Atassi, 1980).

With these considerations in mind, the binding activity of free, unconjugated peptide with [^{3}H]-acetylcholine was tested. As shown in Fig. 2(*a*), the binding activity of free peptide (1.5 μg, 0.51 nmol) with labelled acetylcholine (0.61 nmol) was remarkably high. Peptide 125–147 bound approx. 73% of the labelled acetylcholine (0.87 mol of acetylcholine/mol of peptide) added to the reaction mixture (note the first peak in Fig. 2*a*). This binding activity was reproduced several times with varying amounts of peptide (0.51–34 nmol), and in each case a high binding activity of the synthetic peptide with acetylcholine was observed. In fact, amounts of peptide of 2.5 μg (0.85 nmol) or higher completely removed all of the unbound acetylcholine (0.61 nmol) represented in the second peak (see Fig. 2*a*). In a control experiment, an unrelated haemoglobin α-chain peptide 45–60 was incubated with [^{3}H]acetylcholine under identical conditions. Separation of the reaction mixture on the same Sephadex G-25 column indicated no binding between the control peptide and labelled acetylcholine (results not shown).

Similar binding studies demonstrated the requirement of the disulphide bridge (between cysteine residues 128 and 142) in the synthetic peptide for binding activity to acetylcholine. As shown in Fig. 2(*b*), the treatment of peptide 125–147 with 2-mercaptoethanol for 30 min completely abolished its ability to bind [^{3}H]acetylcholine. In addition, Fig. 2(*c*) shows that the binding activity of the peptide was completely inhibited by preincubation of the peptide with unlabelled acetylcholine (100 mmol excess).

Finally, in binding of [^{3}H]acetylcholine by gel filtration under equilibrium conditions, the monomeric peptide bound 0.94 mol of acetylcholine/mol of peptide as measured from the radioactivity in the peptide peak. On the other hand, the depletion of radioactivity, in the trough area which appears about 20 fractions behind the peptide peak, corresponded to 91% of theoretical. These values are in good agreement with one another and also with the values that are mentioned above.

In the present work, we report the synthesis of a peptide (residues 125–147) around the disulphide bridge 128–142. Early studies (Karlin, 1969, 1980) demonstrated, by affinity labelling with alkylating reagents, the presence of two disulphide bonds which resided near the acetylcholine binding site. Sequence and structural topology studies of the α-chain suggested that only Cys-128, Cys-142, Cys-192, and Cys-193 are located on the extracellular portion of the α-subunit, and are thus accessible to affinity alkylation (Noda *et al.*, 1983*b*,*c*). It has been proposed, therefore, that the acetylcholine binding site is located in a region which includes the residues Cys-128 and Cys-142 (Noda *et al.*, 1982). It was further suggested that, based upon Corey space-filling models of the α-chain, residues Glu-129, His-134 and Asp-138 may also be involved in the binding of acetylcholine.

The studies reported in the present paper have demonstrated that synthetic peptide 125–147 possesses a high binding activity to α-BTX and to acetylcholine. The requirement of the disulphide

Table 2. *Binding of [^{3}H]acetylcholine to adsorbents of synthetic peptide and AChR*

Binding was determined by incubation of 100 μl (packed volume) of Sepharose adsorbents (in triplicate) with [^{3}H]acetylcholine as described in the Experimental section. The total amount of [^{3}H]acetylcholine added to each tube was 5.9×10^6 c.p.m. The values above have been corrected for non-specific binding of the label to Sepharose 4B (740 c.p.m.)

Sepharose adsorbent	[^{3}H]Acetylcholine bound (c.p.m.)
Native AChR	12150 ± 1440
Peptide 125–147	3593 ± 560
Bovine serum albumin control	903 ± 110
Haemoglobin A	780 ± 70
Haemoglobin peptide 1–15	790 ± 90
Myoglobin peptide 137–153	750 ± 130

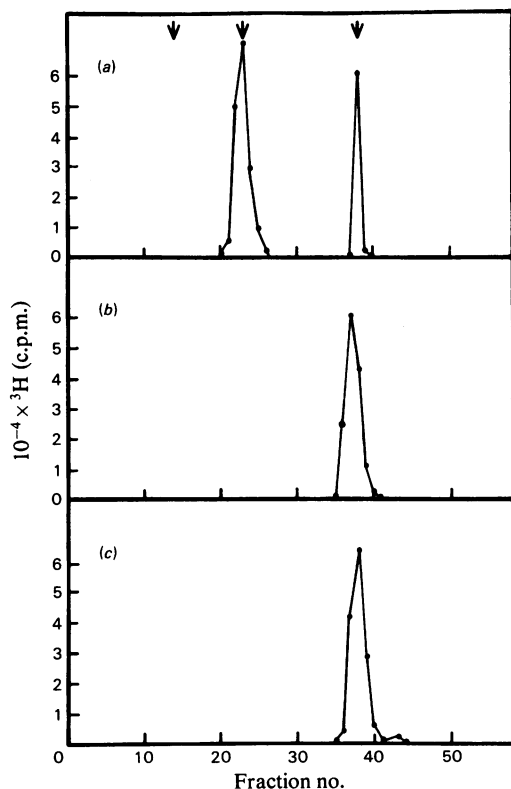

Fig. 2. *Binding of [^{3}H]acetylcholine (3.03×10^6 c.p.m.; 0.61 nmol) to synthetic AChR peptide 125–147 (1.5 μg; 0.51 nmol)*

After being left for 3h at 24°C, the mixture was separated by gel filtration on a column (1.1cm × 36cm) of Sephadex G-25 eluted with PBS and 0.6ml fractions were collected. Aliquots (50 μl) were measured for the presence of [^{3}H]acetylcholine with a scintillation counter. Arrows (↓) indicate the elution of Blue Dextran (fraction 14), free synthetic peptide (fraction 23), and dinitrophenyl-L-alanine (fraction 38). (*a*) Separation of peptide incubated with [^{3}H]acetylcholine only. (*b*) Separation of peptide reduced with 10 μl of 2-mercaptoethanol (30min) followed by incubation with [^{3}H]acetylcholine. (*c*) Elution of peptide incubated with unlabelled acetylcholine (1h, 100mmol excess) followed by incubation with labelled acetylcholine for 3h as above. The second peak (i.e. unbound acetylcholine) in (*a*) above was not present when the amounts of peptide were 2.5 μg (0.85 nmol) or higher.

linkage between Cys-128 and Cys-142 for acetylcholine binding activity is supported by the findings that reduction of this bridge completely destroys the ability of the peptide to bind acetylcholine. These results, however, do not preclude the possibility that additional residues, residing outside the region 125–147, are involved in the binding of the toxin or acetylcholine to the receptor of *T. californica*.

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