Volume 228, number 1, 118–122 FEB 05540 February 1988

Nicotinic acetylcholine receptor: a structural model for α-subunit peptide 188–201, the putative binding site for cholinergic agents

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Received 11 December 1987

A peptide corresponding to amino acid sequence 188–201 of the α-subunit of *Torpedo* AChR binds α-Bgtx. The S-S bridge between Cys 192 and 193 is essential for the binding as Tyr in position 189. The same sequence 188–201 corresponding to human AChR, which instead of Tyr has a Thr in position 189, binds α-Bgtx with a much lower efficiency. Monoclonal antibodies raised against *Torpedo* peptide 188–201 recognize *Torpedo* AChR and antibodies against *Torpedo* AChR recognize peptide 188–201 indicating that the synthetic peptide and the corresponding sequence in the native molecule share some immunological epitopes. With computer graphics and energy refinement a molecular model of this peptide has been elaborated.

Nicotinic acetylcholine receptor; α-Bungarotoxin; Monoclonal antibody; Computer graphics; Molecular modeling; α-Toxin binding site

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is a neurotransmitter receptor which, upon acetylcholine binding, undergoes a conformational change which triggers ion permeability by opening a self-contained cation channel. AChR from vertebrate skeletal muscles and fish electric organs is a membrane complex of four protein subunits α, β, γ and δ of approx. 300 kDa [1,2]. In the functional receptor five subunits (α_2, β, γ, δ) are arranged with pseudo-five-fold symmetry around the central-cation-conducting channel. Of the five subunits only the two α-subunits bind acetylcholine and the specific cholinergic antagonist α-bungarotoxin (α-Bgtx) [1]. Identification of the ACh binding site on the receptor α-subunits is a key point for understanding the molecular mechanism underlying receptor activation.

Chemical modification and affinity labeling studies of both native and detergent-solubilized AChR indicate that a disulphide bridge is present on the α-subunit chains within 10 Å of the acetylcholine binding site [3]. The two Cys building up this intrachain disulphide have been identified as residues 192 and 193 on the basis of affinity labeling studies [4]. Furthermore proteolytic fragments of the α-subunit containing residues 192–193 [5] and synthetic peptides corresponding to the sequences 185–196 [6], 173–204 [5] and 188–201 [7] bind α-Bgtx. All these findings suggest that the sequence flanking residues 192–193, which is one of the few sequences unique to α-subunit, is the major site responsible for the binding of α-Bgtx and cholinergic agents in the α-subunit.

For better understanding the molecular interactions regulating α-Bgtx binding to the α-subunit, we have: (i) synthesized a polypeptide segment corresponding to the amino acid sequence 188–201 of the *Torpedo* and human α-subunits and smaller peptides of the *Torpedo* sequence flanking residues 192–193 (188–194, 191–198 and 195–201) and tested their binding properties (in the presence and absence of chemical modifications of the amino acid side chains) towards α-Bgtx; (ii) used

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Published by Elsevier Science Publishers B.V. (Biomedical Division)

monoclonal antibodies against peptide 188–201 as tools for studying the conformation of peptide 188–201 in solution and in the AChR molecule. The findings obtained and the available chemical and structural information have been employed for the development of a molecular model of this peptide by means of computer graphics and energy refinement.

2. MATERIALS AND METHODS

2.1. *Peptides synthesis and immobilization*

Peptides (table 1) were synthetized manually or using a Beckman system 990 synthesizer by the stepwise solid-phase method proposed by Merrifield [8]. Final deprotection and cleavage of the peptides from the resin was performed by the low HF treatment proposed by Tem et al. [9]. Peptide purification was achieved by gel filtration, ion-exchange and semipreparative reverse-phase liquid chromatography: the purity was ascertained by RP-HPLC in different eluting systems and by amino acid analysis. Both the human and *Torpedo* 188–201 sequence contained an additional Lys and the peptides overlapping the *Torpedo* 188–201 sequence an additional Gly at the NH_2 terminus as spacer in order to improve the yield of immobilization. Peptides were immobilized on CNBr-activated Sepharose 4B (Pharmacia) in 0.1 M bicarbonate buffer, pH 8.3. The amount of coupled peptides (0.5–1.0 μmol of peptide/ml of Sepharose) was determined by amino acid analysis of the HCl hydrolysate.

2.2. *Chemical modification of peptide 188–201 bound to Sepharose*

2.2.1. Cys reduction and carboxymethylation

Sepharose-peptide 188–201 conjugate, suspended in 0.1 M phosphate buffer, pH 8, was at first reduced by treatment with 0.1 M DTT for 30 min at room temperature, then washed with deoxygenated and nitrogen saturated 0.1 M phosphate buffer, pH 6.0, until the washing was negative by Ellman's test. Addition, under nitrogen, of *N*-ethylmaleimide to a final concentration of 0.1 M for 30 min gave a quantitative sulphydryl alkylation as judged by Ellman's test.

2.2.2. Tyr acylation

Tyr acylation was achieved according to Smith [10] treating 1 ml of Sepharose-peptide 188–201 conjugate in 0.1 M phosphate, pH 7.0, with 0.1 ml of a 10% acetic anhydride solution in acetonitrile for 20 min. Then, excess reagent was removed by washing with buffers. Deacylation of the *O*-acetyl Tyr was achieved by treating the Sepharose-peptide *O*-acyl Tyr with 0.04 M hydroxylamine in 0.1 M phosphate, pH 7.0, for 30 min according to Simpson et al. [11].

Table 1

Sequences of the synthetic peptides of the α-subunit of the *Torpedo* californica and human acetylcholine receptor, flanking residues 192–193

Peptide αT 188–201	KVYYTCCPDTPYLDI
Peptide αT 188–194	GVYYTCCP
Peptide αT 191–198	GTCCPDTPY
Peptide αT 195–201	GDTPYLDI
Peptide αH 188–201	KVTYSCCPDTPYLDI

2.2.3. β-Carbonyl amidation

Amidation was performed on the Sepharose-peptide conjugate suspended in 0.01 M phosphate, pH 4.8, by adding a 100-fold excess of both EDC and appropriate amine, buffered at the same pH, overnight: excess reactants were removed by exhaustive washings. Incorporation of glycine methyl ester was complete as judged by amino acid analysis.

2.3. *Toxin purification and labeling*

α-Bgtx was purified from the venom of *Bungarus multicinctus* according to Gotti et al. [12] and was labeled with ^{125}I by the chloramine T method [13] to a specific activity of 30 μCi/nmol.

2.4. Torpedo *AChR purification*

AChR from *Torpedo* membranes was purified as described [14] using an affinity column with *Naja naja siamensis* toxin. Radiolabeling of AChR with ^{125}I was performed by the chloramine T method.

2.5. *α-Bgtx binding to Sepharose-peptides*

2 nmol of various peptide-Sepharose conjugates were incubated with titrated amounts of ^{125}I-α-Bgtx in a final volume of 100 μl phosphate buffer saline (PBS) supplemented with 1% bovine serum albumin (BSA). Incubation was carried out both for 60 min and 24 h at room temperature after which samples were diluted with PBS and centrifuged in a microfuge for 3 min. The beads were washed again twice with PBS and then counted.

2.6. *Monoclonal and polyclonal antibody production and purification*

Monoclonal antibodies (mAbs) against *Torpedo* peptide 188–201 were produced as described [15] using as immunogen peptide 188–201 conjugated to BSA. The conjugate contained approx. 40 molecules of peptide per molecule of BSA. Polyclonal antibodies (PAbs) against *Torpedo* AChR were obtained by immunization of rabbits with 100 μg purified *Torpedo* AChR in complete Freund's adjuvant. Two booster injections were administered two and four weeks later. PAbs were purified by ammonium sulfate precipitation and subsequent DEAE-cellulose ionic-exchange chromatography.

2.7. *Immunoprecipitation of ^{125}I-AChR by mAbs against peptide 188–201*

^{125}I-AChR from *Torpedo* electric organs was incubated with different concentrations of purified mAbs against peptide 188–201 overnight at 4°C, followed by addition of carrier mAb and 50 μl of goat antimouse IgG. Precipitates were washed three times with PBS plus 0.1% Triton X-100 and counted in a γ-counter. In a parallel experiment ^{125}I-AChR was incubated with α-Bgtx for 60 min at 4°C and then with mAbs.

2.8. *Computer modeling*

The statistical predictive method of Chou and Fasman was

applied for establishing the probable secondary structure of the synthesized polypeptide [16]. All the molecular modeling steps were performed on an Evans and Sutherland PS300 graphics system using the program FRODO [17]. Part of the stereochemical refinement process was conducted at the modeling stage applying the procedure of Hermans and McQueen [18] to subfragments of the polypeptide. Final minimizations and energy calculations were done using the program EREF [19]. Atomic coordinates for the C-α positions of α-Bgtx were those available from the Brookhaven Protein Data Bank file.

3. RESULTS AND DISCUSSION

3.1. *Binding of ^{125}I-α-Bgtx to peptides conjugated to Sepharose*

The peptide corresponding to *Torpedo* sequence 188–201 had a maximal binding activity to α-Bgtx. Shortening the peptide length on both sides of residues 192–193 largely decreased the binding capacity. The binding was also reduced when the S-S bridge between Cys 192 and 193 was reduced or alkylated. Restoring the disulphide bridge in mild oxidizing conditions e.g. potassium ferricyanide, restored the binding capacity (fig.1).

In order to verify if other amino acids or side chain residues could be relevant for the toxin binding to peptide 188–201, peptide 188–201-Sepharose conjugate was chemically modified in order to obtain *O*-acyl Tyr peptide, and δ-carboxylamide peptide. A decrease of the binding properties of the peptide was observed when the Tyr was *O*-acylated, while β-carboxyl amidated peptide maintained the binding activity. Restoring the phenolic hydroxyl of Tyr by hydroxylamine, completely restored binding capacity (fig. 1). To further examine the relevance of Tyr for α-Bgtx binding, we have synthetized the peptide corresponding to the sequence 188–201 of the α-subunit of human AChR. This sequence differs from that of *Torpedo* by the substitution of Tyr 189 with a Thr and of Thr 192 with a Ser. This peptide still specifically binds α-Bgtx but this binding is only 5–10% of the binding of α-Bgtx to corresponding peptide of the *Torpedo* sequence (fig. 1). These findings indicate that Tyr in position 189 is really relevant for α-Bgtx binding. These findings also suggest that the sequence 188–201 is not the only site where α-Bgtx binds, as it has been suggested by several authors [20], and/or that the simultaneous binding to other sites in the α-subunit stabilizes or optimizes the binding to the

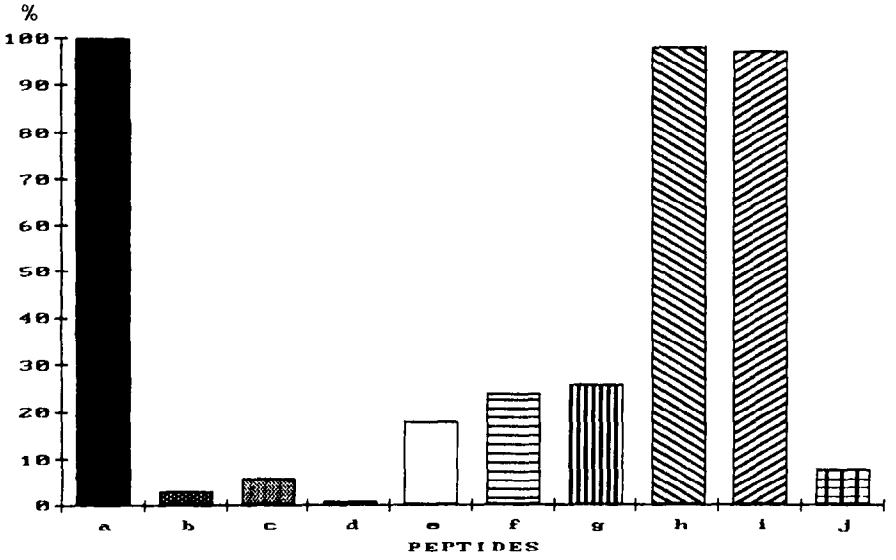

Fig.1. Binding of ^{125}I-α-Bgtx to peptides corresponding to the amino acid sequence 188–201 of *Torpedo* and human α-subunit AChR and to chemically modified and shortened peptides of the 188–201 sequence of *Torpedo* AChR bound to Sepharose. (a) Peptide 188–201 of *Torpedo* AChR in the oxidized form (expressed as 100%); (b) peptide 188–194; (c) peptide 191–198; (d) peptide 195–201; (e) peptide 188–201 reduced with DTT; (f) peptide 188–201 reduced and alkylated with *N*-ethylmaleimide; (g) peptide 188–201 with *O*-acylated Tyr; (h) peptide 188–201 with deacylated *O*-acetyl Tyr; (i) peptide 188–201 with β-carboxyl of aspartic acids and COOH terminal amidated; (j) peptide 188–201 of human AChR.

188–201 peptide. On the basis of these data and of the amino acid sequence we studied the possible molecular models of peptide 188–201.

3.2. *Peptide model*

Consideration of the secondary structure potentials for tetrapeptides in the 188–201 segment indicates a significant propensity for the β-structure [16]. On the other hand, the presence of an accessible disulphide bridge linking two adjacent Cys, followed by a Pro, imposes structural constraints on the conformation attainable by the whole polypeptide. In particular, in order to attain proper S-S covalent bond formation, the peptide bond between adjacent Cys 192 and Cys 193 must adopt the unusual *cis* instead of the *trans* conformation [21, 22]. Isomerization of the peptide bond may also occur at Pro residues, such as Pro 194. In this case, because of the five membered side chain ring constraint, the *trans* Pro structure is favoured by only 2 kcal/mol compared to the *cis* isomer. Under particular circumstances, such as occurrence of a -Pro-Pro- segment within longer polypeptides or proteins, one of the two residues has a *cis* peptide bond [23].

The 188–201 polypeptide was model built according to these criteria as extended structure, with chain reversal at Cys-192–Cys-193, and taking into consideration the two alternative structures for the Pro 194 peptide bond. Independent refinement of the two models indicated that lower overall energy can be attained by the structure displayed in fig.2, in which both 192–193 and 193–194 are *cis* peptide bonds. This structure could be refined to an overall energy of -27 kcal/mol, while isomerization of Pro 194 (and subsequent rearrangement of the following residues) yielded a polypeptide conformation which could be refined to -14 kcal/mol. As can be seen from fig.2 the conformation proposed for the polypeptide is extended but not regular, especially at the turn where considerable contraints are present. Nevertheless the phi-psi backbone

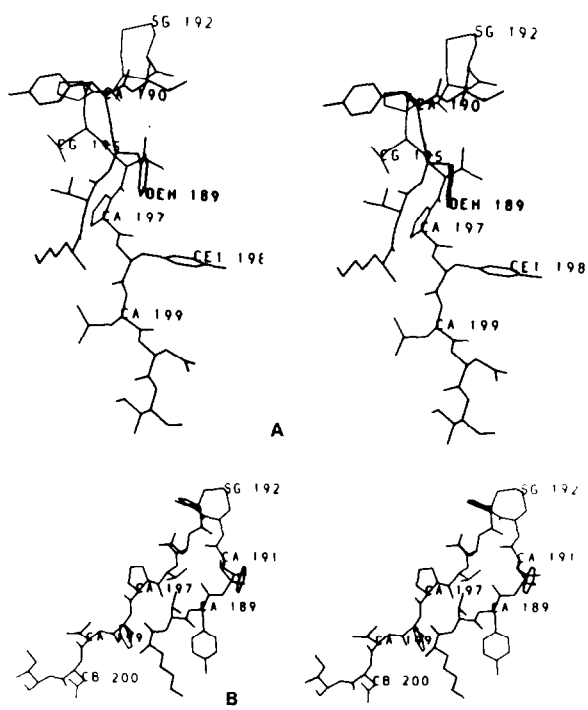

Fig.2. Two computer generated stereo pictures of the modeled 188–201 peptide; the two views are approx. 90° apart. The S-S disulphide is in the upper part of the picture. Amino acid residues are labeled according to their sequence position.

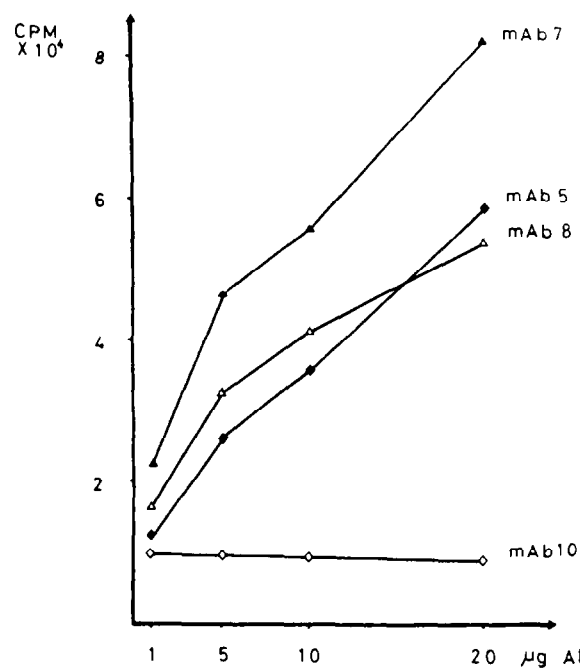

Fig.3. Immunoprecipitation of ^{125}I-*Torpedo* AChR by monoclonal antibodies raised in mouse against peptide 188–201 bound to KLH. ^{125}I-*Torpedo* AChR was incubated, overnight at 4°C with the indicated concentrations of purified mAbs against peptide 188–201 (mT7, mT5, mT8) or control mAb (mT10). The complexes were then precipitated by addition of 50 μl of goat and antimouse IgG. Precipitates were washed twice and counted.

conformational parameters fall well within the accepted limits of the Ramachandran plot [23]. Three hydrogen bonds connect the two antiparallel strands, but only Tyr-189-O–Thr-196-N shows adequate donor-acceptor orientation.

The conformation proposed is consistent with a protein loop pointing to the receptor surface at the site of chain reversal. This is in accordance with solution studies showing the ready accessibility of the Cys-192–Cys-193 disulphide to reducing reagents [24]. The structure proposed here for the 188–201 peptide of the α-subunit of the nicotinic acetylcholine receptor however does not allow one to extend the prediction to its binding mode to the surfaces of antagonists such as snake neurotoxins, for which detailed molecular models are available [25]. Despite convincing evidence that the receptor peptide binds to the concave face of α-neurotoxins, and that specific toxin residues have been identified as functionally important for the binding [26], we could model at least two peptide-neurotoxin complexes which equally well accounted for the existing data.

3.3. *Antibodies binding studies*

Concerning the question of the structure adopted by the free 188–201 peptide compared to that present in the native receptor, experiments with antibodies showed that PAbs raised in rabbits against *Torpedo* AChR and a mAbs (Mc1 T1) raised against *Torpedo* AChR recognize, in an ELISA assay, peptide 188–201 (not shown). Furthermore three mAbs against peptide 188–201 are able to recognize *Torpedo* AChR (fig.3). However, these mAbs were unable to precipitate *Torpedo* AChR which had been preincubated with α-Bgtx indicating that the antigenic site recognized by mAbs was hidden by α-Bgtx (not shown). These data suggest that the synthetic peptide 188–201 (i) can have, also in solution, a conformation similar to that shown in the native AChR since it is recognized by PAbs against AChR and mAbs against the peptide recognize AChR; (ii) is a relevant part of the binding site of α-Bgtx in the α-subunit since Abs against the synthetic peptide do not recognize AChR when it has bound α-Bgtx.

In conclusion, our results on the binding, structure and immunological conformation of peptide 188–201 indicate that this amino acid sequence is relevant for binding cholinergic agents also on the native AChR and that this sequence cannot be altered without loss of toxin binding.

Acknowledgements: This work was supported in part by a CNR Grant within the Strategic Project 'Neurobiologia'. We thank Dr G. Mazzola for the helpful discussions during the experiments.

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