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Acetylcholine interactions with tryptophan-184 of the α-subunit of the nicotinic acetylcholine receptor revealed by transferred nuclear Overhauser effect

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Acetylcholine interactions with three genetically engineered fusion proteins containing peptides from the nicotinic acetylcholine receptor were studied by 1D and 2D nuclear magnetic resonance methods The three proteins were *Torpedo* α184–200, *Torpedo* α186–198, and human α183–204 of the acetylcholine receptor fused to the first 323 residues of the *E coli* protein trpE Nuclear Overhauser effect studies revealed interactions of bound acetylcholine with tryptophan-184 present in the *Torpedo* α184–200, and the human α183–204 sequences These interactions are between the $N(CH_3)_3^+$ and CH_3 groups of acetylcholine with the aromatic protons of tryptophan The appearance of these cross-peaks indicates a distance of less than 5 Å between tryptophan and the bound ligand, however, direct contact has yet to be proven

Acetylcholine receptor, Acetylcholine interaction, Ligand binding site, Nuclear Overhauser effect, Nuclear magnetic resonance (NMR)

1. INTRODUCTION

The binding site of the nicotinic acetylcholine receptor (AChR) for the snake α-neurotoxins has been located on the receptors α-subunits (for reviews see [1]) Numerous studies using short peptide sequences, either synthetic or recombinant, have assigned the neurotoxin binding site to the α170–210 region [2–8] Recently, we have shown that the α183–204 sequence from various species contains the cholinergic binding site as well [9,10]. The search for discrete amino acids that are reponsible for interaction with the bound ligand has employed various methods and a multidisciplinary approach These include site directed mutagenesis [11,12], sequence modifications of synthetic peptides [13–15], affinity labeling [16–20], and comparison of ligand binding to several different sequences so as to determine the importance of defined amino acids to binding [8,12,21]. Such studies have implicated a number of aromatic residues involved in the binding process

The analysis of ligand binding by nuclear magnetic resonance (NMR) has proven to be especially effective for low affinity interactions [9,10] In the present study we describe experiments in which transferred nuclear Overhauser effect (tNOE) [22] has been applied This method is particularly advantageous in cases where the protein is large and the concentration of the relevant complex is low Transferred 1D and 2D NOE experiments on several ACh/recombinant-peptide complexes have identified trp-184 as potentially being involved in ACh binding

Abbreviations BTX, α-bungarotoxin, H, human, NOE nuclear Overhauser effect, NOESYPH, phase-sensitive 2D NOE, T, *Torpedo*

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2 MATERIALS AND METHODS

2 1 *Materials*

The preparation of recombinant cholinergic binding sites corresponding to the sequences α184–200, α186–198 (kindly provided by Dr Rachel Moscovitz) from the *Torpedo* AChR (Tα184–200, Tα186–198), and to the α183–204 sequence from the human AChR (Hα183–204, kindly provided by Dr Bella Ohana), have been previously described [7,12] In addition a negative control protein (pATH) a random peptide sequence fused to truncated trpE, has been used [7] Their amino acid sequences are listed in Table I

2 2 *NMR measurements*

1D and 2D spectra were acquired with a Bruker AM360-WB spectrometer equipped with an Aspect 3000 computer Protein and ACh concentrations were 0 1–0 3 mM and 7 mM, respectively Phase sensitive 2D-NOE experiments [23,24] were done with a few mixing times (τ_m) including τ_m = 0 calibration (see the following section) Spectral widths of these experiments were 8 ppm α-Bungarotoxin (BTX) was added in excess (2–3-fold over the amount of binding sites) to the ACh/protein solution and an additional phase sensitive 2D-NOE experiment was carried out

3 RESULTS

A phase-sensitive 2D-NOE experiment was performed on the ACh/Tα184–200 complex. The experiment was repeated with several mixing times τ_m=0, 0 2 s, 0 25 s and 1 6 s Fig. 1 shows the contour plot of the 2D map of ACh bound to Tα184–200 (τ_m =0.2 s). All peaks in the spectrum are negative as expected for

Table I

The amino acid sequence of the four recombinant peptides, showing their tryptophan distribution

		184W	187W
Tα184–200	- W K H W V Y Y T C C P D T P Y L D - - - -	+	+
Tα186–198	- - - H W V Y Y T C C P D T P Y - - - - - -	-	+
Hα183–204.	G W K H S V T Y S C C P D T P Y L D I T Y H	+	-
Path	G D P L E S T C S P S L S M I S C Q T - - -	-	-

macromolecules with $\omega\tau_c >> 1$ or for a ligand bound to such molecules. Note the appearance of two peaks on the diagonal in the aromatic region (6 40 and 7.42 ppm) which have cross-peaks between each other. In addition cross-peaks were observed between the 7 42 ppm resonance and the acetyl CH_3 group and between the 6 40 ppm resonance and both the acetyl CH_3 and the $N(CH_3)_3^+$ groups of the ACh. As ACh does not contain any aromatic residues these two resonances must be attributed to the protein. These peaks and their cross-peaks with ACh disappeared in the absence of ACh or upon addition of BTX. Furthermore, cross-peaks among the ACh protons in the presence of BTX changed their magnitude but preserved their sign, indicating residual non-specific binding. This result indicates that the source of these cross-peaks is the specific interactions between the ACh and the Tα184–200 domain which is the binding site of BTX Moreover, analysis of ACh in the presence of the pATH protein (see Fig 2 and Table I) was performed. A complete absence of peaks in the aromatic region is seen on the 2D map, while all cross-peaks within the ACh resonances are still negative indicating the residual binding to the pATH protein. One should note that neither the random peptide nor the trpE protein which constitute the pATH protein contain any tryptophan residues

Chemical shift data from NMR spectra of polypeptides such as lysozyme [25] attribute the resonances with δ = 6 40 and 7.42 ppm to tryptophan. In contrast to tyrosine, the chemical shift of tryptophan residues in proteins are highly variable In the lysozyme all resonances of the three tyrosine residues fall within the range of 6.71–7.24 ppm On the other hand, the aromatic signals of three of the tryptophans which were assigned span the range of 6 28–6 83 ppm for the C(5)

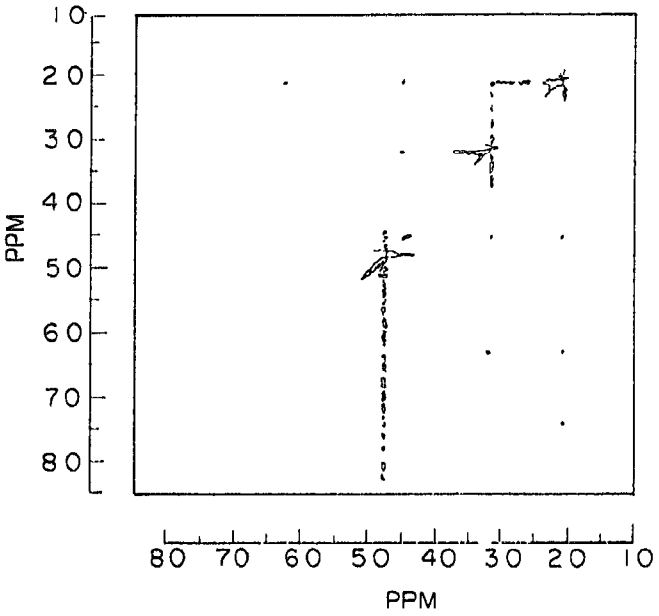

Fig. 1 Contour plot of a phase-sensitive transferred 2D-NOE spectrum of ACh bound to Tα184–200 demonstrating ACh interacting with resonances in the aromatic region HOD peak at 4 8 ppm serves as a reference Tα184–200 and ACh concentrations are 0 13 mM and 7 mM, respectively. Mixing time is 0 2 s, relaxation delay is 12 s and the spectral window is 2700 Hz.

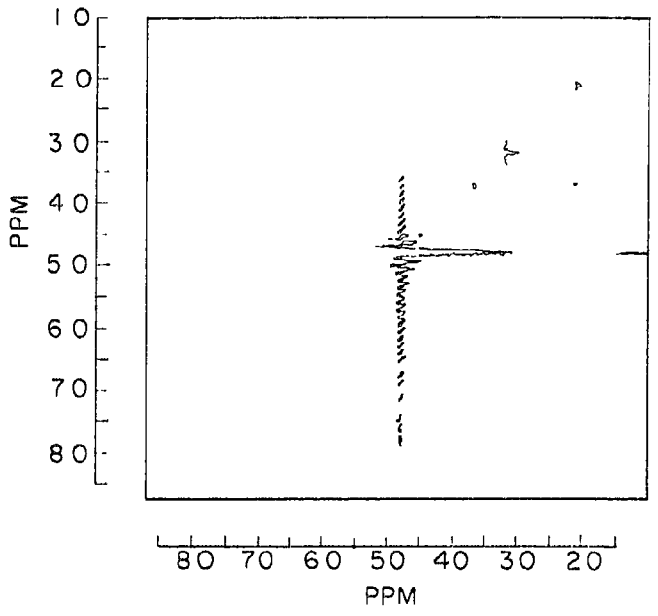

Fig 2 Contour plot of a phase-sensitive transferred 2D-NOE spectrum of ACh interacting with the recombinant protein pATH demonstrating lack of an aromatic region HOD peak at 4 8 ppm serves as a reference pATH, and ACh concentrations are 0 27 mM and 7 mM, respectively Mixing time is 0 2 s, relaxation delay is 12 s and the spectral window is 2700 Hz

proton and 6.76–7.75 for the C(2), C(4), C(6) and C(7) protons. Thus the resonance that gives rise to NOE fall well within this range In an effort to confirm our hypothesis, based on the comparison of Tα184–200 with the pATH protein and chemical shift data, that tryptophan is responsible for the aromatic resonances observed in the t2D-NOE experiments we have executed the same experiments with ACh interacting with the Tα186–198 and Hα183–204 sequences. The K_d values of ACh binding to T184–200, T186–198 and H183–204 proteins were previously determined by selective T_1 NMR measurements and are 1 93 [9], 3 5 [26] and 2.7 [10] mM, respectively The tryptophan contents of the two, together with that of the Tα184–200 sequence, are shown in Table I

Fig. 3 shows the contour plot of the transferred 2D-NOE of ACh (τ_m = 0.3 s) interacting with the Hα183–204 The experiment was repeated with τ_m = 0, 0.15 s, 0.2 s and 0 3 s. The human sequence exhibits the same aromatic region though at a slightly different chemical shift (6 22 and 7.27 ppm). In contrast to these two maps no aromatic region is seen in the Tα186–198 map (Fig 4). It should be noted that also in these experiments all peaks in the spectrum are negative indicating that the NOE is due to transfer from ACh bound to these proteins Trp-184 is present only in the *Torpedo* α184–200 and human sequences.

4. DISCUSSION

There are several approaches to assess the structure of the ACh binding site of the receptor; examples are: (i) point mutagenesis evaluating the effects of specific changes on the binding constants, (ii) affinity labeling, analyzing the products of chemical modifications or photoaffinity labeled substrate, (iii) NMR, which has been used to determine the conformation of ACh bound to the intact receptor [27] and ligand interactions with recombinant binding sites 1D en 2D tNOE used in the present communication have enabled the specific identification of Trp-184 as interacting with bound ACh. This has been achieved by comparing t2D-NOE spectra of three distinct recombinant binding sites The unique peaks, 6 40 and 7 42 appear, however, only when the binding site is occupied with ACh. Lack of the agonist or its displacement BTX abolished these peaks (and of course their corresponding cross-peaks). Whether or not intimate van der Waals contacts or other direct molecular contact between ACh and Trp-184 exist is still an open question These observations indicate that the indole ring of Trp-184 assumes a novel conformation as a result of the presence and close proximity of the $N(CH_3)_3^+$ and CH_3 groups of ACh thus resulting in cross-peaks as well One can conclude that the spatial distance is less than 5 Å. Note that lack of Trp-184 in the Tα186–198 sequence does not lead to non-specific binding The involvement of tryptophan in ligand binding has been previously suggested by others [6,12] Our

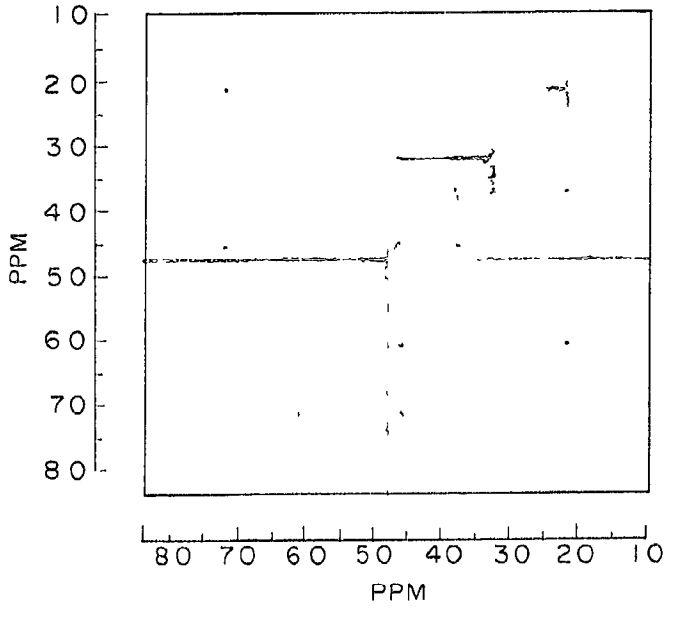

Fig 3 Contour plot of a phase-sensitive transferred 2D-NOE of ACh bound to Hα183–204 demonstrating the respective aromatic region Peaks are referenced to the HOD peak at 4 8 ppm ACh and peptide concentrations are 7 mM and 0 27 mM, respectively Mixing time is 0 2 s, relaxation delay is 14 s and the spectral window is 2500 Hz

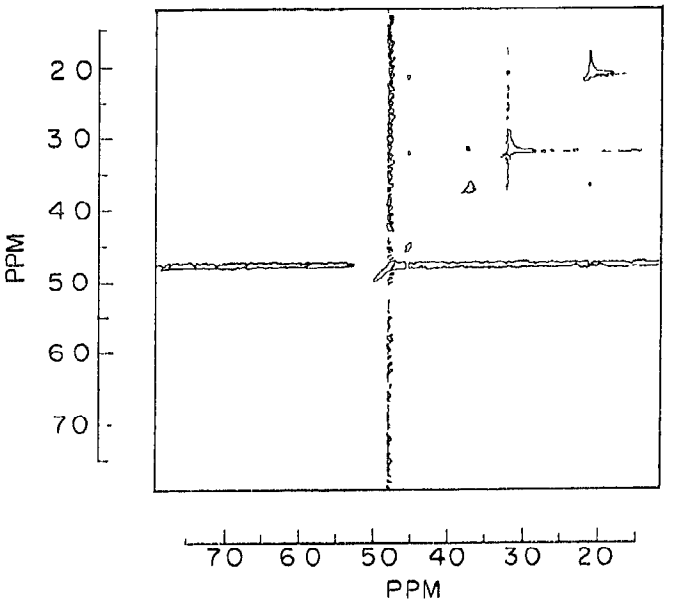

Fig 4 Contour plot of a phase sensitive transferred 2D-NOE of ACh bound to Tα186-198 demonstrating the absence of the respective aromatic region HOD peak at 4 8 ppm is the reference ACh and Tα186-198 concentrations are 7 mM and 0 27 mM, respectively Mixing time is 0 2 s, relaxation delay is 14 s and the spectral window is 2600 Hz

results support and expand this action by providing direct quantitative evidence for Trp-184 interactions with ACh. Such interactions are also in line with the possible stabilizing attraction between a quaternary ammonium group and the π electrons of electron-rich aromatic systems as proposed for ACh binding [28]

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