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ARTICLE in FEBS LETTERS · NOVEMBER 1991

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# Acetylcholine interactions with tryptophan-184 of the $\alpha$ -subunit of the nicotinic acetylcholine receptor revealed by transferred nuclear Overhauser effect

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Received 19 July 1991, revised version received 23 August 1991

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*  $\alpha$ 184–200, *Torpedo*  $\alpha$ 186–198, and human  $\alpha$ 183–204 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*  $\alpha$ 184–200, and the human  $\alpha$ 183–204 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  $\alpha$ -neurotoxins has been located on the receptors  $\alpha$ -subunits (for reviews see [1]). Numerous studies using short peptide sequences, either synthetic or recombinant, have assigned the neurotoxin binding site to the  $\alpha$ 170–210 region [2–8]. Recently, we have shown that the  $\alpha$ 183–204 sequence from various species contains the cholinergic binding site as well [9,10]. The search for discrete amino acids that are responsible 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.

## 2 MATERIALS AND METHODS

### 2.1 Materials

The preparation of recombinant cholinergic binding sites corresponding to the sequences  $\alpha$ 184–200,  $\alpha$ 186–198 (kindly provided by Dr Rachel Moskovitz) from the *Torpedo* AChR (T $\alpha$ 184–200, T $\alpha$ 186–198), and to the  $\alpha$ 183–204 sequence from the human AChR (H $\alpha$ 183–204, 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 ( $\tau_m$ ) including  $\tau_m = 0$  calibration (see the following section). Spectral widths of these experiments were 8 ppm.  $\alpha$ -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/T $\alpha$ 184–200 complex. The experiment was repeated with several mixing times  $\tau_m = 0, 0.2$  s, 0.25 s and 1.6 s. Fig. 1 shows the contour plot of the 2D map of ACh bound to T $\alpha$ 184–200 ( $\tau_m = 0.2$  s). All peaks in the spectrum are negative as expected for

**Abbreviations:** BTX,  $\alpha$ -bungarotoxin; H, human; NOE, nuclear Overhauser effect; NOESYPH, phase-sensitive 2D NOE; T, *Torpedo*.

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

		184W	187W
T $\alpha$ 184-200	-WKHWVYYTCCPDTPYLD----	+	+
T $\alpha$ 186-198	---HWVYYTCCPDTPY-----	-	+
H $\alpha$ 183-204.	GWKHSVTYSCCPDTPYLDITYH	+	-
Path	GDPLESTCSPSLSMISCQT---	-	-

macromolecules with  $\omega\tau_c \gg 1$  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  $\text{CH}_3$  group and between the 6.40 ppm resonance and both the acetyl  $\text{CH}_3$  and the  $\text{N}(\text{CH}_3)_3^+$  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 T $\alpha$ 184-200 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  $\delta = 6.40$  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)

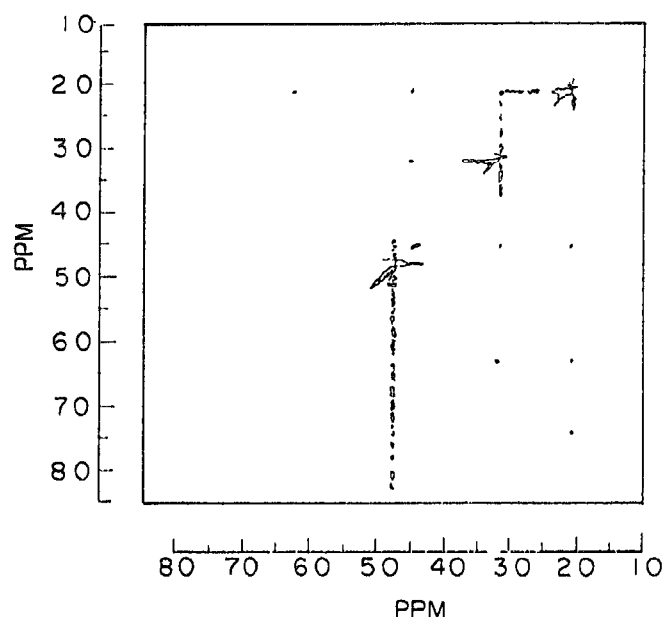


Fig. 1 Contour plot of a phase-sensitive transferred 2D-NOE spectrum of ACh bound to T $\alpha$ 184-200 demonstrating ACh interacting with resonances in the aromatic region. HOD peak at 4.8 ppm serves as a reference. T $\alpha$ 184-200 and ACh concentrations are 0.13 mM and 7 mM, respectively. Mixing time is 0.2 s, relaxation delay is 12 s and the spectral window is 2700 Hz.

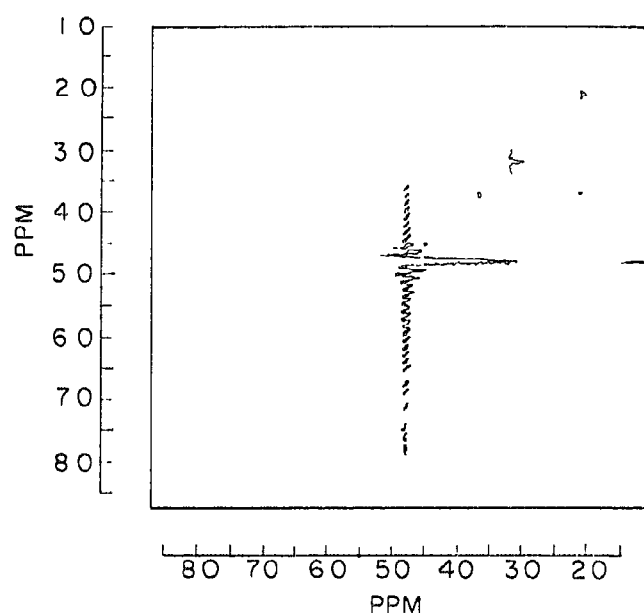


Fig. 2 Contour plot of a phase-sensitive transferred 2D-NOE spectrum of ACh interacting with the recombinant protein pATH demonstrating lack of an aromatic region. HOD peak at 4.8 ppm serves as a reference. pATH, and ACh concentrations are 0.27 mM and 7 mM, respectively. Mixing time is 0.2 s, relaxation 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 T $\alpha$ 184–200 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 T $\alpha$ 186–198 and H $\alpha$ 183–204 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 T $\alpha$ 184–200 sequence, are shown in Table I.

Fig. 3 shows the contour plot of the transferred 2D-NOE of ACh ( $\tau_m = 0.3$  s) interacting with the H $\alpha$ 183–204. The experiment was repeated with  $\tau_m = 0, 0.15$  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 T $\alpha$ 186–198 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*  $\alpha$ 184–200 and human sequences.

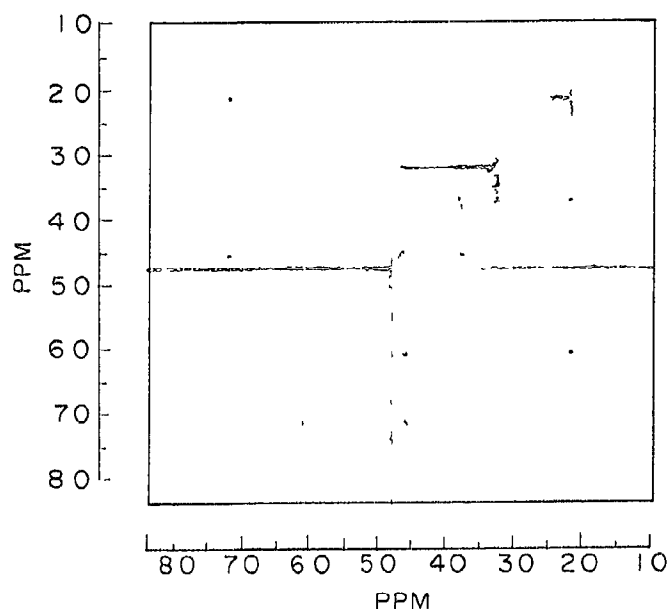


Fig. 3 Contour plot of a phase-sensitive transferred 2D-NOE of ACh bound to H $\alpha$ 183–204 demonstrating the respective aromatic region. Peaks are referenced to the HOD peak at 4.8 ppm. ACh and peptide concentrations are 7 mM and 0.27 mM, respectively. Mixing time is 0.2 s, relaxation delay is 14 s and the spectral window is 2500 Hz.

#### 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 and 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 T $\alpha$ 186–198 sequence does not lead to non-specific binding. The involvement of tryptophan in ligand binding has been previously suggested by others [6,12]. Our

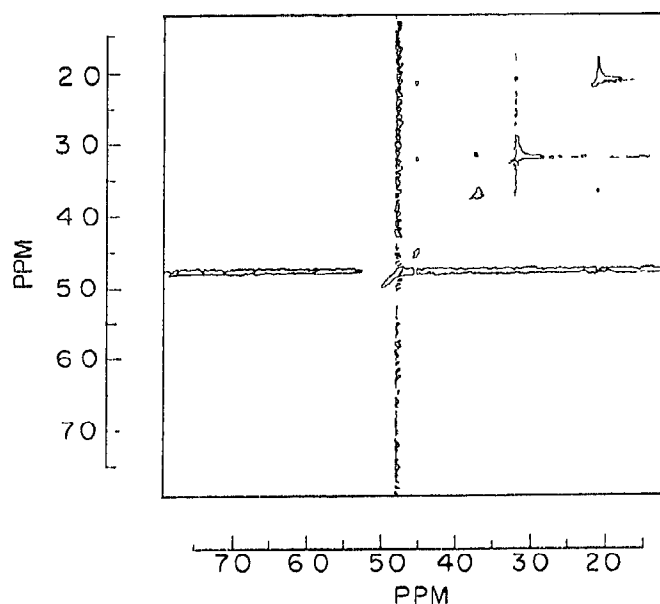


Fig. 4 Contour plot of a phase-sensitive transferred 2D-NOE of ACh bound to T $\alpha$ 186–198 demonstrating the absence of the respective aromatic region. HOD peak at 4.8 ppm is the reference. ACh and T $\alpha$ 186–198 concentrations are 7 mM and 0.27 mM, respectively. Mixing time is 0.2 s, relaxation 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  $\pi$  electrons of electron-rich aromatic systems as proposed for ACh binding [28]

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