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Direct Measurement of Agonist Binding to Genetically Engineered Peptides of the Acetylcholine Receptor by Selective T_1 NMR Relaxation†

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ABSTRACT: Interactions of four ligands of the nicotinic acetylcholine receptor with genetically engineered peptides have been studied by NMR. A recombinant cholinergic binding site was prepared as a fusion protein between a truncated form of the bacterial protein trpE and a peptide corresponding to the sequence α184–200 from the *Torpedo californica* receptor. This construct binds α-bungarotoxin while the trpE protein alone does not, and thus serves as a negative control [Aronheim, A., Eshel, Y., Mosckovitz, R., & Gershoni, J. M. (1988) *J. Biol. Chem. 263*, 9933–9937]. In this study agonist binding to α184–200 is demonstrated by monitoring the T_1 relaxation of the ligand's protons in the presence and absence of the recombinant binding site. This binding is specific as it can be competed with α-bungarotoxin. Quantitative analyses of such competitions yielded the concentration of binding sites, which corresponded to 3.3% and 16.5% of the total protein, for partially purified and affinity-purified α184–200 constructs, respectively. The K_D values for the binding of acetylcholine, nicotine, *d*-tubocurarine, and gallamine to the affinity-purified construct were 1.4, 1.4, 0.20, and 0.21 mM, respectively, while K_D's with the nontoxin binding protein were all above 10 mM. Thus, this is a direct demonstration that the toxin binding domain α184–200 may comprise a major component of the cholinergic agonist site.

With the advent of recombinant DNA technologies much has been learned about the structure of the nicotinic acetylcholine receptor (AChR)[1] [for recent reviews, see Popot and Changeux (1984), Hucho (1986), McCarthy et al. (1986), and Lentz and Wilson (1988)]. Since 1982, the complete amino acid sequences of a wide variety of AChR's have been published (Ballivet et al., 1983; Numa et al., 1983; Boulter et al., 1985, 1988; Bossy et al., 1988). From these studies, not only has the subunit composition of the AChR been confirmed, but it has become clear that all the receptor subunits thus far analyzed are common to one gene family. Furthermore, new types of subunits have been discovered (Takai et al., 1985). The "next step" in studying the structure and function of the

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[1] Abbreviations: ACh, acetylcholine; AChR, nicotinic acetylcholine receptor; BTX, α-bungarotoxin; GA, gallamine; NMR, nuclear magnetic resonance; TC, *d*-tubocurarine.

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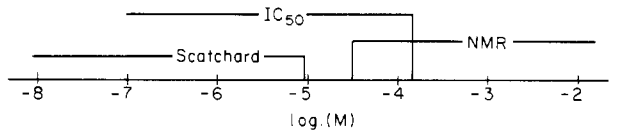

FIGURE 1: Range of different binding assays. The typical biochemical determination of ligand binding constants relies on the effective separation of bound from free ligand after the system has reached equilibrium. Such Scatchard analyses are precise in measuring $K_D \leq 10^{-5}$ M. Pharmacological competition of the strong binding ligand with weaker ones can extend the range of measurable affinity through correlation with the IC_{50}. The NMR approach, based on rapid chemical exchange of bound and free ligand and averaging, allows one to study binding of low-affinity interactions with greater precision.

AChR thus requires the correlation of discrete peptide sequences with their respective functions. A search for channel elements, membrane-traversing sequences, the major immunogenic region associated with myasthenia gravis, and the ligand binding sites have been undertaken.

The study of the ligand binding site has been particularly productive. Initially, it was suggested that the area of residues α151–210 harbors a major element of the α-neurotoxin binding site (Neumann et al., 1985, 1986a). This conclusion was based on a systematic protein blot analysis of the receptor; i.e., identification of toxin binding proteolytic fragments of the α-subunit and mapping of these with sequence-specific antibodies [for summary, see Gershoni (1987a,b)]. Subsequently, it has been repeatedly demonstrated that synthetic peptides, or recombinant proteins expressing the area of α180–200, contain essential aspects of the α-neurotoxin binding site (Wilson et al., 1985; Mulac-Jericevic & Atassi, 1986; Neumann et al., 1986b; Barkas et al., 1987; Gershoni, 1987a,b; Gotti et al., 1987; Ralston et al., 1987; Aronheim et al., 1988; Radding et al., 1988).

The common denominator to all these studies is that, despite the various experimental manipulations performed, α-bungarotoxin (BTX) continues to bind even fragments of the binding site with measurable affinities. This is not the case, however, for the cholinergic agonists, and so one can only assume that α180–200 might be involved in agonist binding as well. The reason for this is that biochemical binding assays are inadequate for the evaluation of low-affinity ($K_D \geq 0.1$ mM) interactions (see Figure 1). In contrast, NMR techniques, due to the averaging properties brought about by rapid chemical exchange, allow one to obtain quantitative information concerning agonist–receptor complexes with low binding constants. As was pointed out by Valensin et al. (1982), the selective T_1 relaxation technique is particularly sensitive to binding of small ligands to macromolecules and can be developed for the determination of binding constants (Kushnir & Navon, 1983). This technique was recently applied to the measurement of the binding of various agonists to the intact AChR of *Torpedo californica* (Behling et al., 1988; Navon et al., 1988).

In the present study we have analyzed a number of synthetic and recombinant peptides for their binding of cholinergic agonists and antagonists. It was demonstrated that the sequence α184–200 specifically binds the neurotransmitter acetylcholine (ACh), its agonist nicotine, and the antagonists *d*-tubocurarine (TC) and gallamine (GA).

Materials and Methods

Materials. Synthetic peptides corresponding to α1–20, α126–143, and α184–200 were synthesized by the Merrifield solid-state method at The Weizmann Institute. They were partially purified and were better than 85% in the main peak as esimated by HPLC. Oligonucleotides corresponding to

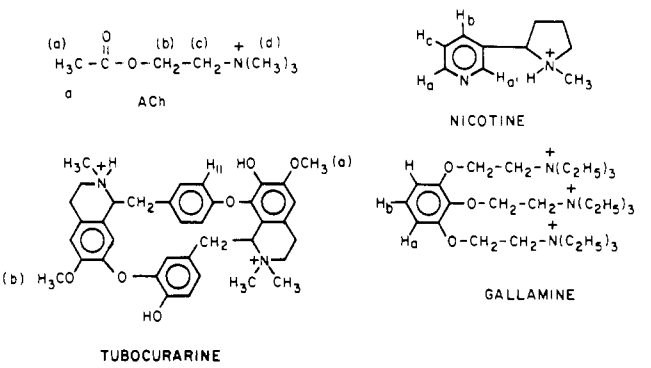

FIGURE 2: Structure formulas of four cholinergic ligands used in this study. Assignments are indicated for those protons referred to in the text and in the following figures.

defined sequences of the α-subunit of AChR from *Torpedo californica* were inserted into the pATH2 expression vector (Dieckmann & Tzagoloff, 1985). The plasmids were used to transform *Escherichia coli* K-12 HB101 strain. The transformed bacteria were grown under inductive conditions to produce a major fusion protein (R4137), which consists of the *T. californica* AChR sequence α184–200 fused to the carboxy terminus of a truncated trpE protein. Induction of bacteria was performed as previously described (Gershoni, 1987a,b.). In principle, the bacteria were cultured on M9 medium supplemented with casamino acids, and 3β-indoleacrylic acid, an inhibitor of de novo biosynthesis of L-tryptophan. Enrichment of the fusion protein was accomplished by sonication and differential centrifugation as described (Aronheim et al., 1988). Nicotine and TC were purchased from Fluka Chemie AG, ACh was from Sigma, and GA was from Aldrich. They were all used without further purification, and the assignment and relevant protons is shown in Figure 2.

Affinity Purification. Induced bacteria were pelleted and washed in 10 mM phosphate buffer (pH 7.4) and resuspended to a concentration of 20 OD_{600}/mL in buffer containing 1 mM EDTA, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.5 mM NaN_3, and 10 mM Tris-HCl, pH 7.4 (buffer A). The suspension was sonicated and centrifuged to obtain the enriched fraction of R4137 as described previously (Aronheim et al., 1988). This fraction was then incubated overnight at 4 °C with a *Naja naja siamensis* α-cobratoxin column (Sigma Chemicals). The beads were initially pretreated in 0.2 M acetic acid for 30 min and equilibrated with buffer A containing 1% Triton X-100 (w/v) (Mosckovitz & Gershoni, 1988). After the incubation, the mixture was filtered on sintered glass and washed with 100 mL of buffer A plus 1% Triton X-100 and then with 50 mL of buffer A + 0.1% Triton X-100. Bound fusion protein was eluted by incubating the resin with 10 mL of 1 M carbamylcholine in water for 4–5 h at 4 °C. The eluant was dialyzed at 4 °C for 24 h against buffer A plus 0.1% Triton X-100.

Biochemical Binding Assays. Toxin binding and competition assays were performed as previously described (Aronheim et al., 1988). R4137 protein or truncated trpE protein derived from the pATH2 vector (a nontoxin binding protein and, so, a negative control) was incubated with ^{125}I-labeled BTX with or without various amounts of competing ligands. After 30-min incubation, the free radioactive BTX was separated from the bound by vacuum filtration through Zetabind charge-modified nylon membranes (Cuno Specialty Materials, Inc., Meriden, CT). The concentration of bound ligand was then calculated. The IC_{50} values, i.e., the concentration of competing ligands that reduces BTX binding to 50% of its

maximal level, was also determined. Nonspecific binding was determined by adding a 1000-fold excess of nonradioactive BTX to the reactions.

NMR Measurements. Relaxation rates were determined on a Bruker AM360-WB spectrometer equipped with an ASPECT 3000 computer. Selective and nonselective T_1's were measured by the inversion–recovery pulse sequence, where the desired resonance in the selective experiment was inverted with a long weak pulse from the decoupler. T_1 values were calculated with the Bruker software.

K_D Determination. In a ligand–receptor system where the ligand (L) is present in a large excess over the receptor binding sites (R) concentrations, so that the fraction of bound ligand is small, the ligand relaxation rate $1/T_1$ is described by

$$1/T_1 = 1/T_{1f} + \sum_i f_i/(T_{1i} + \tau_i) \quad (1)$$

T_{1f} is the relaxation time of the free ligand and f_i, T_{1i}, and τ_i are the fractions, relaxation times, and the lifetimes of the various bound states. We obtain the specific effect of the binding site of interest (i = b) by subtracting from $1/T_1$ the relaxation rate after addition of the specific inhibitor BTX, $1/T_{1n}$. Thus, the net effect due to site b is given by

$$1/T_{1p} = 1/T_1 - 1/T_{1n} \quad (2)$$

Defining

$$f = [RL]/[L]_0 \qquad K_D = [R][L]/[RL] \quad (3)$$

where $[L]_0$ is the total ligand concentration and [RL] is the complex concentration, eq 1 takes the form

$$[R]_0 T_{1p} = ([L]_0 + K_D)(T_{1b} + \tau_b) \quad (4)$$

$[R]_0$ is the total receptor binding site concentration. Thus, a plot of $[R]_0 T_{1p}$ vs $[L]_0$ has a slope of $T_{1b} + \tau_b$, and K_D is determined from the intercept on the X axis. The measurements were done under the following conditions: 2 mg of the synthetic peptide was dissolved first in 0.1 mL of DMSO-d_6 and then added to 0.4 mL of phosphate buffer (pH 7.4), 0.025 M, in D_2O; 5 mg of the partially purified (or 2 mg of the affinity purified) recombinant peptide was directly dissolved in 0.02 M phosphate buffer in D_2O. Ligands were added in increments, and T_1 was measured twice at each concentration. The temperature was maintained at 25 ± 1 °C.

Determination of the Number of Binding Sites. BTX was added in increments to a solution containing either the partially purified or the affinity-purified R4137 together with 7 mM ACh. T_1 was measured for each BTX concentration.

Results

In this study antagonist binding to bacterially expressed toxin binding proteins was examined. Specifically, the trpE expression vector pATH2 was used. In this system fusion proteins consist of chimeric structures of which the first 323 residues are derived from bacterial protein trpE. Following 1–323, one can insert sequences of interest. Two fusion proteins were prepared: (i) R4137, which introduces the sequence PGIEGRWKHWVYYTCCPDTPYLD (derived from α184–200 of the α-subunit of the AChR) immediately after 1–323 of trpE; (ii) pATH2 protein as a negative control, which contains the random peptide sequence PGDPLESTCSPSLSMISCQT following 1–323. The most potent α-neurotoxin, BTX, binds R4137 (corresponding to α-184–200) with an affinity of 5×10^{-8} M. Although this affinity is 3–4 orders of magnitude less than that observed for the native receptor, it is still easily analyzed in a standard filtration binding assay. The association of less efficient ligands such as TC and GA

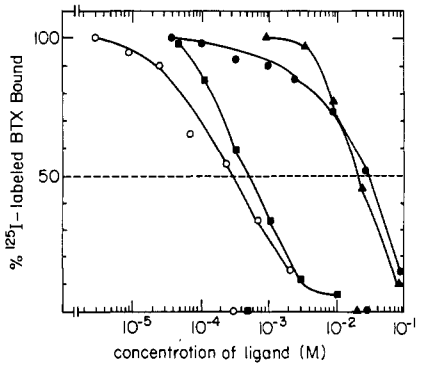

FIGURE 3: Competition of BTX binding to R4137. ^{125}I-Labeled α-BTX (2×10^{-8} M) was mixed with ever-increasing concentrations of *d*-tubocurarine (■), gallamine (○), acetylcholine (▲), and NaCl (●). The mixtures were incubated with equal amounts of R4137 for 30 min at 25 °C, and the net amount of bound radioactive toxin was determined.

Table I: T_1^{sel} Values (s) of Agonists in the Presence of Recombinant Peptides

ligand[a]	free	R4137[b]	pATH	+BTX[c]
ACh				
CH_3-a	4.80	2.10	3.40	3.67
$N(CH_3)_3$-d	1.87	1.20	1.40	1.55
nicotine				
Haa′	5.33	0.58	2.19	2.44
H_b	3.53	0.54	1.70	1.98
H_c	4.62	0.92	1.90	2.20
TC				
OCH_3-a	0.93	0.28	0.75	0.84
OCH_3-b	0.98	0.24	0.78	0.86
H-11	0.80	0.15	0.64	0.74
GA				
H_a	0.83	0.21	0.68	0.78
H_b	1.74	0.23	1.43	1.67

[a] For assignment, see Figure 2. [b] Affinity purified. [c] Addition of 2–3-fold excess of BTX to the R4137/ligand solution.

can only be inferred via competition assays deriving the IC_{50} values in each case; both of which have a value of 5×10^{-4} M (see Figure 3). The IC_{50} values for agonists such as carbamylcholine or ACh are beyond the reliable detection level of these assays where nonspecific salts, such as sodium chloride, compete equally well (Haggerty & Froehner, 1981; Aronheim et al., 1988). We therefore decided to apply the selective T_1 technique (Valensin et al., 1982; Navon et al., 1988; Behling et al., 1988) to the study of agonist binding to R4137. It was necessary to determine whether or not agonist binding to R4137 could be detected at all. For this, selective T_1 relaxation times were measured in the presence or absence of R4137 (see Table I). Excessive BTX was also added to R4137 as a control to demonstrate the specific displacement of ACh (Table I, last column). R4137 significantly decreased the relaxation times while the pATH2 protein, which does not bind toxin, had a much smaller effect, which closely resembles that of the BTX control. Further validation of the specificity of the effect of R4137 was shown by titrating the binding sites with BTX. BTX titration of ACh bound to R4137 causes a gradual decrease in the R_1 ($R_1 = 1/T_1$) of ACh. A plot of R_1 vs [BTX] for partially purified R4137 is shown in Figure 4. The curve obtained is a descending line that levels off at the point where all the binding sites are saturated with BTX and thus no longer specifically effect the ACh T_1 relaxation. This point gives the binding site concentration. For 0.27 mM partially purified R4137 (Figure 4), the value was 8.9 μM, corresponding to 3.3% of the total protein concentration, while for 0.109 mM affinity-purified protein (data not shown) the value was 18

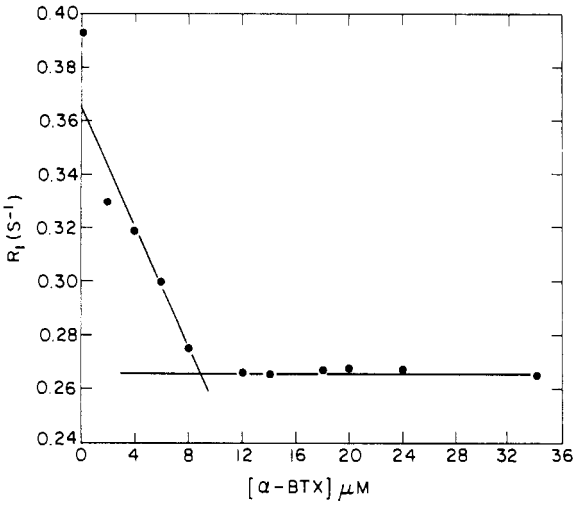

FIGURE 4: Determination of the concentration of binding sites. ACh (7 mM) was mixed with partially purified R4137 (0.27 mM). As BTX was added in increments, the R_1^{sel} ($1/T_1^{sel}$) of the acetyl group of ACh decreased up to the point where BTX displaced all bound ACh.

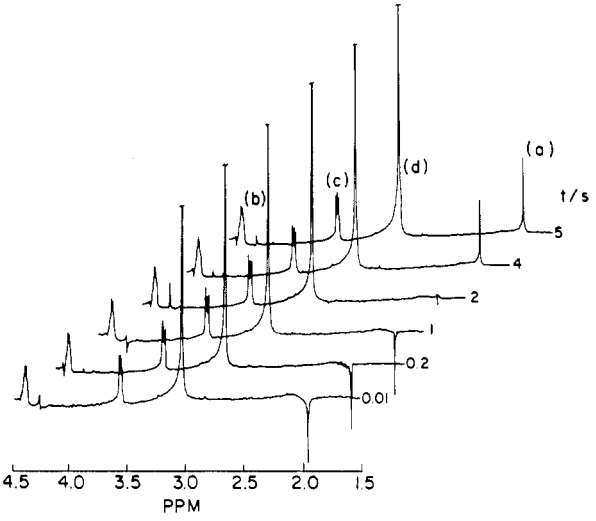

FIGURE 5: Demonstration of a selective inversion–recovery experiment, where the acetyl group of ACh designated (a) is inverted. For assignment of peaks a–d, see Figure 2.

μM, being 16.5% of the binding sites.

Determination of K_D. Selective relaxation was monitored for the ligands bound to the recombinant peptide R4137 and the pATH2 protein. A demonstration of a selective T_1 experiment for ACh is shown in Figure 5. By adding ligand in increments and measuring T_1 for every ligand concentration, we calculated the K_D with eq 4 (see Materials and Methods). Typically, the concentration of the recombinant protein was 0.2–0.3 mM. The initial ligand concentration was 0.1–0.2 mM, and increments of added ligand varied in the range of a few millimolar. Addition of BTX raised the T_1 value, though not to its initial value, due to nonspecific binding of the ACh to the bacterial protein. K_D values for the ligands tested are given in Table II. Figure 6 demonstrates the difference in K_D values of ACh bound to R4137 and the pATH protein.

From the above experiments it is clear that R4137 specifically binds agonists such as ACh and nicotine. In view of the many reports of synthetic peptides that bind α-neurotoxins (see introduction), it was of interest to test the binding of ACh to a synthetically prepared peptide corresponding to α184–200. Such a peptide was found to be rather insoluble; however, analyses could be performed in 20% DMSO. Under such conditions ACh and other ligands were found to bind to synthetic α184–200 with affinities similar to those found for R4137 (Tables III and IV). The specificity of the binding was shown by comparing the effect of the other synthetic

Table II: K_D Values (mM) for Ligand Binding to Recombinant Peptides

ligand[a]	R4137[b]	pATH
ACh (CH_3-a)	1.4	16
nicotine (H_b)	1.4	14
TC (OCH_3-b)	0.2	19
GA (H_a)	0.21	11
GA[c] (H_a)	0.27	15

[a] For assignment, see Figure 2. [b] Affinity purified. [c] Plus 20% DMSO.

Table III: T_1^{ns} Values (s) of Agonists in the Presence of Synthetic Peptides

ligand[a]	free	+α184–200 peptide	+nonbinding peptides	+BTX[d]
ACh				
CH_3-a	4.34	1.70	3.54[c]	3.80
nicotine				
Haa′	4.77	0.77	1.62[c]	1.98
H_b	2.93	0.52	1.53[c]	1.81
TC				
OCH_3-a	0.88	0.35	nd	0.81
OCH_3-b	0.90	0.32	0.74[b]	0.84
GA				
H_a	0.74	0.15	0.54[b]	0.69
H_b	1.33	0.18	0.88[b]	1.15

[a] For assignment, see Figure 2. [b] α126–143. [c] α1–20. [d] Addition of 2–3-fold excess of BTX to the α184–200/ligand solution.

Table IV: K_D Values (mM) for Ligand Binding to Synthetic Peptides

ligand[a]	α184–200	α126–143	α1–20
ACh			
CH_3-a	2.70	12	12
nicotine			
Haa′	1.80	10	10
H_b	1.68	10	11
TC			
OCH_3-b	0.11	12	nd
GA			
H_a	0.23	12	11
H_b	0.24	11	nd

[a] For assignment, see Figure 2.

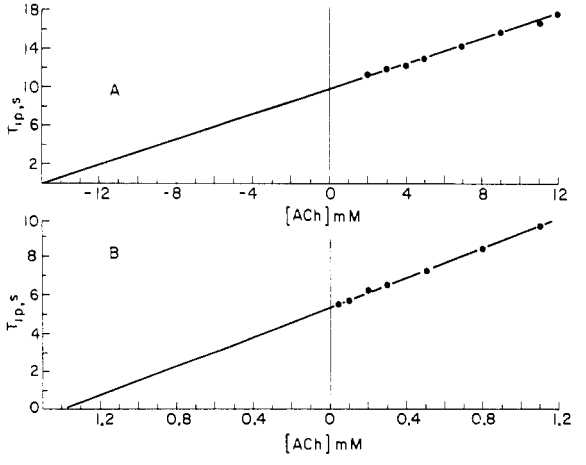

FIGURE 6: Determination of K_D's of ACh binding to recombinant peptides with eq 4 (see Materials and Methods). (A) pATH2. (B) R4137. Note the different scales on the X axis. Peptide concentration was 0.27 mM in both experiments, while ACh concentration varied: 1–12 mM in (A) and 0.1–1.5 mM in (B).

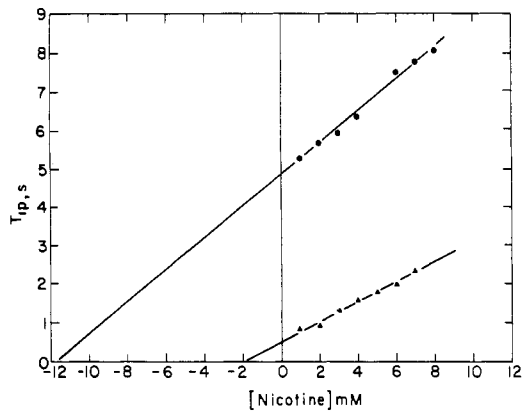

FIGURE 7: Determination of K_D of nicotine binding to synthetic peptides with eq 4 (see Materials and Methods): α1–20 (●); α184–200 (▲). Peptide concentration was 1.8 mM, and nicotine concentration was in the range of 1–8 mM.

peptides corresponding to α1–20 and α126–143 (Figure 7). Surprisingly, however, was the fact that the total amount of measurable binding sites was extremely low ($\simeq$0.05%). This fact implies that whereas qualitatively the peptide α184–200 binds cholinergic ligands, only relatively few of the peptide molecules can actually assume a functional configuration. This phenomenon appears to be common in other studies as well [see, for example, Figure 4A in Wilson and Lentz (1988) in which only 6.3×10^{-3}% of the material examined actually binds toxin].

Discussion

The identification of a functional domain in a given protein is still a rather complicated task. The problem would be best solved were one able to simply excise out and isolate a given sequence that independently continued to function. Most often, however, this is not feasible, and many postulates therefore rely on negative results, such as loss of activity after site-directed mutagenesis. The study of the α-neurotoxin binding site of the AChR has, however, complied nicely with the production of positive results.

Fragments of the AChR can be produced that represent appreciably less than 10% of the receptor, yet continue to bind BTX reasonably well. Such mimic binding sites have recently been shown to act as molecular decoys and can neutralize α-neurotoxin in vivo (Gershoni & Aronheim, 1988). Despite the fact that the area α184–200 convincingly binds antagonists, there have been others that place the "physiologically relevant" agonist binding site elsewhere (McCormick & Atassi 1984; Luyten 1986; Kosower, 1987). The basis for the latter conclusions has been either theoretical, by computer modeling, or experimental, from toxin binding to synthetic peptides.

In this study we have deliberately preferred to use recombinant fusion proteins expressing relatively short peptides rather then simply examine chemically synthesized peptides. Indeed, synthetic peptides are by now routinely prepared and have been applied in the study of the cholinergic binding site as well. However, the sequence on hand is particularly difficult to work with. It is relatively water insoluble, and thus, most of the work done by others has demonstrated solid-phase assays rather than the study of binding of peptides in solution. For the purpose of this study, the peptides required 20% DMSO for their solubilization, which obviously does not represent the natural milieu. Furthermore, the absolute specific binding activity has been found to be exceedingly low. Whereas the peptide resolved by HPLC is >85% one peak (not shown), less than 0.1% of its mass actually binds. Similar results have been reported by others, yet the explanation for this is still unclear.

In cases where conclusions pertaining to low-affinity agonist binding is concerned, competition experiments have been necessary as no direct binding assay has been found suitable. Therefore, in this paper we have addressed the issue of the cholinergic agonist site in a most direct manner, looking for positive binding to receptor sequences in solution. In order to accomplish our goal, we employed the selective T_1 relaxation technique to the examination of recombinant fusion protein. The T_1 technique is straightforward and well worked out. It lends itself to the analysis of low-affinity ligand binding to large molecules. The affinities found by NMR for the biochemically measurable ligands compare extremely well with the values derived from the IC_{50} experiments here and previously. In an attempt to compare our findings with those using synthetic peptides, we found that only α184–200 bound agonists or antagonists and that α126–143 [previously postulated to be a ligand binding domain by Noda et al. (1982)] was inactive as well α1–20. The analyses of the synthetic peptides were complicated by the requirement of solubilization in 20% DMSO. This concentration however did not appear to be deleterious in assays of R4137 (comparing the last two entries in Table II and unpublished data).

Most important is the fact that, by use of the T_1 technique in combination with the recombinant R4137, it has been possible to demonstrate direct binding of ACh and nicotine to this previously identified toxin binding site. It is hard to conceive that such small ligands could interact specifically with more than only a few properly situated amino acids residues. By systematically modifying the recombinant binding sites and continuing to apply NMR analysis, the identification of these residues as well as their configuration and contribution to ligand recognition will be realized.

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