Probing the Agonist Binding Pocket in the Nicotinic Acetylcholine Receptor: A High-Resolution Solid-State NMR Approach

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ABSTRACT: Acetylcholine, the agonist for the nicotinic acetylcholine receptor, has been observed directly when bound specifically to its binding site in the fully functional receptor-enriched membranes from *Torpedo nobiliana*. High-resolution solid-state, magic angle spinning ^{13}C NMR methods have been used to observe selectively $N^+(^{13}CH_3)_3$ acetylcholine bound in as few as 20 nmol of receptor binding sites, against a background of natural abundance membrane resonances and excess acetylcholine in free solution. The specificity of the binding has been demonstrated to be pharmacologically significant through the use of the competitive inhibitor α-bungarotoxin which selectively displaces and prevents binding of acetylcholine to the membrane-bound receptor. The chemical shift assigned to $N^+(^{13}CH_3)_3$ acetylcholine in solution and crystalline solid is 53.9 ± 0.04 ppm, and it changes by 1.6 ppm ($p < 0.05$) for agonist when bound specifically in the receptor binding site. Through the use of computer simulations of chemical shifts carried out on acetylcholine bound to the acetylcholinesterase, we propose that the cause for this change is the presence of aromatic side chains lining the receptor binding site. It is suggested that the binding of acetylcholine to the nicotinic acetylcholine receptor is mediated primarily through the interaction of the quaternary ammonium group of the acetylcholine with the π bonded systems in the aromatic side chains. Longitudinal relaxation time measurements show that the residency time for the acetylcholine observed in DDCP experiments is long (>200 ms) with respect to the longitudinal relaxation time of other assignable resonances within the spectrum from the lipid and protein and confirms that the acetylcholine is protein-associated, and not free in solution or nonspecifically bound.

The ligand-gated ion channel, the nicotinic acetylcholine receptor (nAcChoR) is a member of the four transmembrane helix superfamily of receptors which includes GABA, glycine, and the 5-HT_3 receptors. Structurally the nicotinic acetylcholine receptor is composed of five glycosylated subunits ($\alpha_2\beta\gamma\delta$) with a total molecular mass of 280 kDa (*1*). Electron diffraction studies have been used to resolve the structure of the receptor at 9 Å and how it is conformationally altered upon the binding of acetylcholine to the synaptic surface (*2*).

High resolution (≪9 Å) structural studies of the nicotinic acetylcholine receptor have been hampered by the absence of crystals suitable for X-ray crystallography. Additionally, conventional solution state NMR[1] cannot be employed in structural studies of membrane proteins since the complexes in which they exist are too large to produce the high-resolution spectra necessary for detailed structural studies. However, solid-state NMR methodology (*3*, *4*) is now providing valuable information relating to ligand−protein interactions (for reviews, see refs *5*−*7*). Since the anisotropic magnetic interactions leading to spectral broadening possess a $(3\cos^2\theta - 1)$ dependence, by rapidly rotating the sample at the magic angle (θ) of 54.73° with respect to the magnetic field (magic angle spinning, MAS) it is possible to average these interactions and generate high-resolution-like NMR spectra from membrane systems. In addition, MAS can be combined with methods such as cross polarization (CP) to improve signal-to-noise in carbon-13 spectra by making use of the protons with their high abundance and favorable relaxation characteristics (*8*, *9*). We have used CP-MAS to observe selectively ligands and substrates constrained in their binding sites in membrane proteins (*6*, *7*, *10*). This selectivity arises because the isotropic motions experienced by ligands in solution completely average the $^1H-^{13}C$ dipolar interactions necessary for cross polarization to occur, making them invisible in a CP-MAS experiment. In contrast, the immobile nature of ligand that is bound to the protein leads to efficient cross polarization, and hence detection by NMR is possible, even in the native fluid state of the membranes (*10*).

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[1] Abbreviations: CP, cross polarization; DDCP, dephase delayed cross polarization; DFP, diisopropylfluorophosphate; $\Delta\nu_{1/2}$ line width at half-height; MAS, magic angle spinning; M_o, initial magnetization; M_z, magnetization after time t; NMR, nuclear magnetic resonance; T_{1z}, spin lattice relaxation.

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Here it is shown that using CP-MAS NMR methodology, the pharmacologically specific binding of carbon-13 enriched acetylcholine to the nicotinic acetylcholine receptor can be observed. The significant chemical shift changes for bound agonist, when compared to solid agonist, or excess ligand in aqueous solution either in the absence or presence of membrane preparations, indicate that the binding site in the receptor is most likely to be lined with aromatic residues. The observed shifts are similar to those which have been predicted by computer simulations carried out using crystallographic data of quaternary ammonium compounds bound to the acetylcholinesterase. In addition, we show through the use of relaxation phenomena, that it is possible to give an estimate for the residency time of the ligand while bound to the nicotinic acetylcholine receptor.

EXPERIMENTAL PROCEDURES

Synthesis of Carbon-13 Labeled Acetylcholine. Choline labeled at the *N*-methyl position was synthesized from ethanolamine mixed with a slight excess of ^{13}C methyliodide (Sigma) and mixed at room temperature in ethanol. The product was subsequently recrystallized from hot ethanol. The choline iodide was subsequently acetylated using acetic anhydride, in pyridine in the presence of the catalyst (dimethylamino)pyridine (*11*). The reaction was left overnight at room temperature. Filtration followed by recrystallization from a hot saturated solution of ammonium perchlorate in ethanol gave acetylcholine perchlorate. Identity and purity were checked by ^1H NMR (1.9, 3.0, 3.5, 4.35 ppm) and electrospray mass spectroscopy ($F_w = 147$), with an overall yield >20%. Acetylcholine perchlorate was added to the membrane preparation as small aliquots (no more than 25 μL of liquid were added to the pellet during any experiment) from a concentrated stock solution in 10 mM phosphate buffer (pH 7.0), until the concentration of agonist was equivalent to the number of receptor binding sites as determined using a dansyl-choline displacement fluorescence assay (*12*).

Preparation of Enriched Acetylcholine Receptor Membranes. Membranes rich in the nicotinic acetylcholine receptor were purified according to the method of Sobel (*13*) from *Torpedo nobiliana.* Membranes were resuspended in 20 mM phosphate buffer (pH 7.0) to a protein concentration of 1 mg mL^{-1} and treated with 10 mM diisopropylfluorophosphate (DFP) to inhibit acetylcholinesterase activity. Specific inhibition of the nicotinic acetylcholine receptor was achieved by incubating membranes (4 °C; 24 h) with α-bungarotoxin at a concentration of between 5 and 10 times that of agonist binding sites prior to treatment with DFP. Membrane pellets were prepared by ultracentrifugation (4 °C; 80000*g*; 1 h) of membrane suspension and contained typically 0.8 nmol binding site per milligram of protein, as determined using a dansyl-choline displacement fluorescence assay (*12*). About 50 mg of protein was subsequently loaded into a 7 mm MAS NMR rotor and sealed using Kel-F caps for subsequent NMR studies.

NMR Methods. CP-MAS NMR was carried out at 100.63 MHz for carbon-13 (400.13 MHz for protons) using a Bruker MSL-400 spectrometer. A spinning speed of 2500 ± 2 Hz was used for all samples and their temperature maintained at 283 ± 1 K using bearing air. A proton field strength of 50 kHz was applied for cross polarization and decoupling of crystalline samples. The proton field strength was reduced to a level sufficient to provide adequate decoupling for membrane samples, which resulted in an attenuation by 60% for acquisition. Proton-decoupled carbon-13 MAS spectra were acquired with a 5 μs carbon pulse with decoupling during acquisition reduced to a level sufficient to provide adequate decoupling for membrane samples while minimizing sample heating. Samples were referenced to a secondary standard of adamantane whose low-field peak is given as 37.6 ppm.

The dephased delayed cross polarization (DDCP) pulse sequence used to determine the rates of ligand exchange (*14*) is similar to those used for spectral editing in crystalline samples. Initially magnetization is converted to transverse magnetization by a non-selective $\pi/2$ pulse. Halfway through the initial rotor period, a π pulse with opposite phase refocuses any chemical shift and accounts for any error in pulse length. After this initial rotor period, magnetization is restored along the longitudinal axis by means of a non-selective $\pi/2$ pulse. During this initial rotor period, transverse magnetization associated with the immobile membrane-bound components, including receptor and bound ligand, have sufficient time to dephase due to their short T_2 while ligands in free solution retain their in-phase magnetization. The subsequent mixing time allows the ligand to exchange with that in free solution. The subsequent observation by conventional cross polarization allows the selective detection of newly bound ligand provided that the rate of exchange is faster than the recovery in magnetization due to T_{1z} relaxation. Therefore, a lower limit can be placed on the residency time for carbon-13 enriched acetylcholine which is bound to the receptor.

Computer Simulations. The expected perturbation in carbon-13 chemical shift due to the presence of aromatic systems has been simulated on an SGI-Indy, using simulations available from M. P. Williamson (*15*, *16*) based on the Haugh−Mallion model (*17*). The predictions made were based on the position of aromatic resonances within the crystal structure of acetylcholine bound to the acetycholinesterase (*18*) (Brookhaven Protein Data Bank Name 2ACE).

RESULTS AND DISCUSSION

CP-MAS Spectra of Acetylcholine Perchlorate. CP-MAS spectra of unlabeled acetylcholine perchlorate gave five resonances which could be assigned to five magnetically inequivalent sites (Figure 1A). The resonances have been assigned on the basis of those from solution and shielding considerations; CO (172.1 ppm, a), OCH_2 (64.5 ppm, b), NCH_2 (59.2 ppm, c), $N(CH_3)_3$ (53.9 ppm, d), and CH_3 (20.0 ppm, e). In later studies on membrane systems, the $N(CH_3)_3$ intensity has been further enhanced through the specific incorporation (to ∼98%) of carbon-13 at these sites.

Acetylcholine Bound to the Nicotinic Acetylcholine Receptor. To samples of nicotinic acetylcholine-enriched receptor membranes containing 40 nmol of binding site was added 20 nmol of $N^+(^{13}CH_3)_3$ acetylcholine. Upon the addition of acetylcholine, the receptor adopts its desensitized state, with a K_D for acetylcholine of 10^{-9} M (*19*). Under such conditions the bulk of the acetylcholine is bound to the nicotinic acetylcholine receptor.

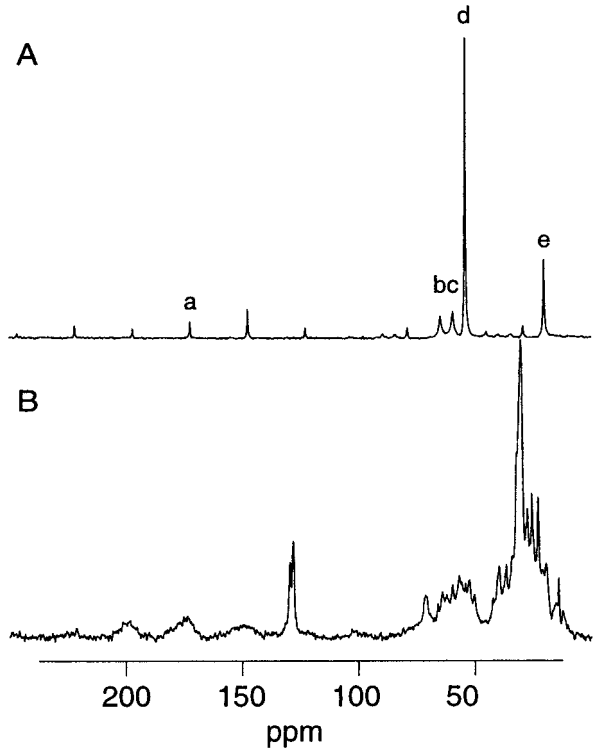

FIGURE 1: CP-MAS NMR carbon-13 spectrum of unlabeled acetylcholine perchlorate, recorded with 1 ms cross polarization and averaged over 32 acquisitions (A). Nicotinic acetylcholine receptor-rich membranes containing 40 nmol of binding site to which 20 nmol of $N^+(^{13}CH_3)$ acetylcholine has been added, recorded with 1 ms cross polarization and averaged over 8600 acquisitions at 283 K (B). Data processed with 20 Hz linebroadening.

The cross polarization spectrum of nicotinic acetylcholine receptor membranes is shown (Figure 1B). The spectrum is dominated by the natural abundance carbon-13 from phospholipid acyl chains between 10 and 40 ppm. The short cross polarization time employed here (1 ms) enables the immobile protein with its efficient cross polarization characteristics to be observed selectively giving a broad spread of resonances between 45 and 70 ppm (*10*). Upon this envelope, resonances which can be attributed to lipid headgroups are resolved with line widths which approach the homogeneity of the magnet ($\Delta\upsilon_{1/2} \sim 15$ Hz). The glycerol backbone of the phospholipids gives rise to the envelope appearing between 70 and 80 ppm. Resonances appearing between 125 and 130 ppm are attributable to the unsaturated vinyl groups within the phospholipid acyl chains. Carbonyl groups are observed at 170 to 180 ppm, and due to their highly anisotropic nature they give rise to sidebands spaced at intervals of 2500 Hz (the sample spinning speed) from the isotropic envelope.

The resonance from $N^+(CH_3)_3$ acetylcholine in solution observed in a conventional solution state NMR spectrum (data not shown) and in the solid observed by CP-MAS NMR (Figure 1A) gives resonances at 53.90 ± 0.04 ppm with reference to adamantane (see Materials and Methods). When added to receptor-rich membranes (50 mg of total protein), at a 100-fold excess over the number of binding sites for acetylcholine, a narrow resonance ($\Delta\upsilon_{1/2} \sim 25$ Hz) at 53.90 ± 0.04 ppm dominates the spectrum acquired using proton-decoupled, single-pulse, (not cross-polarized) ^{13}C NMR (Figure 2A).

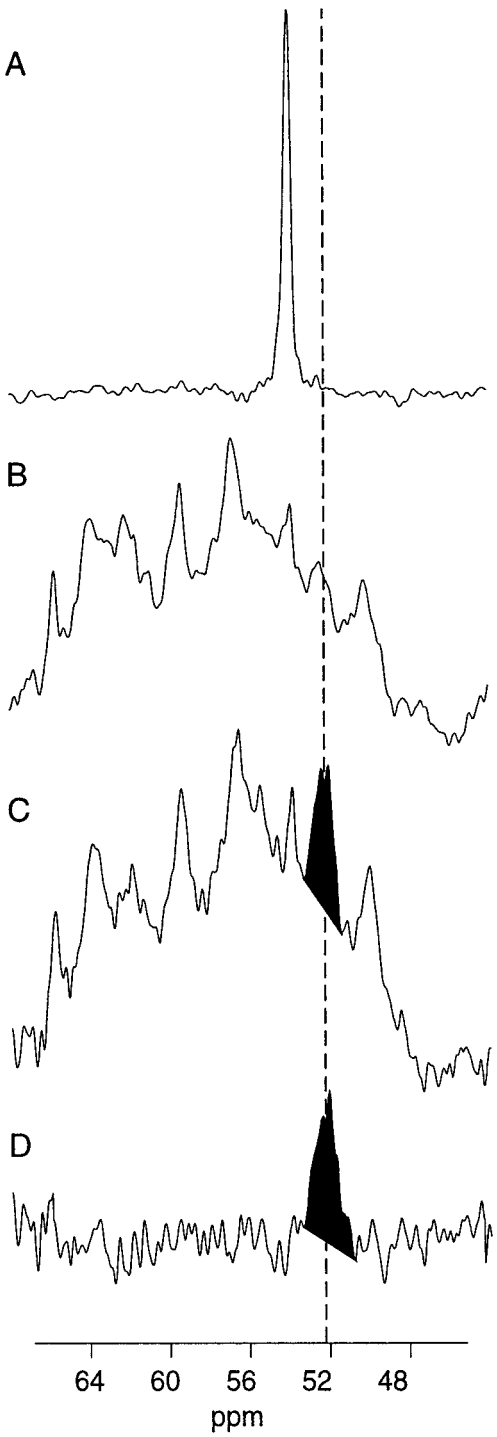

FIGURE 2: Carbon-13 MAS spectra (68−44 ppm) of acetylcholine receptor-rich membranes containing 40 nmol of acetylcholine receptor binding sites. Proton-decoupled spectra acquired with excess $N^+(^{13}CH_3)$ acetylcholine added and averaged over 4000 acquisitions at 283 K. Data processed with 40 Hz linebroadening (A). As in (A) but recorded using CP-MAS in the absence of $N^+(^{13}CH_3)$ acetylcholine (B), upon the addition of 40 nmol of $N^+(^{13}CH_3)$ acetylcholine (C). Difference spectrum resulting from the subtraction of B from C (D). Spectra acquired with a 1 ms cross polarization and averaged over 8600 acquisitions at 283 K, data processed with 20 Hz linebroadening. Shaded area represents intensity due to bound $N^+(^{13}CH_3)$ acetylcholine, and dotted line indicates the position for the bound $N^+(^{13}CH_3)$ acetylcholine.

In the absence of added $N^+(^{13}CH_3)_3$ acetylcholine, using CP methods, the broad envelope (48−64 ppm) of protein resonances are observed (Figure 2B) upon which a signal

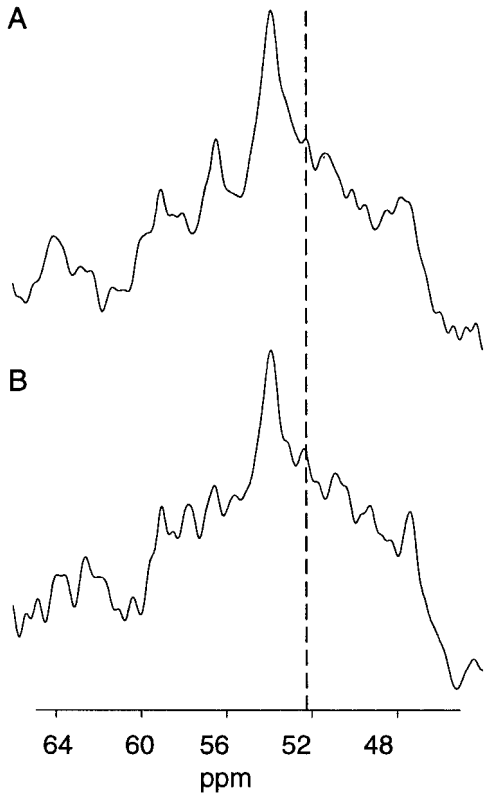

FIGURE 3: CP-MAS NMR carbon-13 spectra (68−44 ppm) of nicotinic acetylcholine receptor-rich membranes containing 40 nmol of receptor binding site as in Figure 2 but with added α-bungarotoxin in the absence of $N^+(^{13}CH_3)$ acetylcholine (A) and in the presence of 40 nmol of $N^+(^{13}CH_3)$ acetylcholine (B). Spectra recorded with 1 ms cross polarization and averaged over 8600 acquisitions at 283 K. Data processed with 20 Hz linebroadening. Dotted line located at same position as in Figure 2.

from acetylcholine is observed (Figure 2C) when an equimolar concentration of the ^{13}C-labeled agonist is added (40 nmol in the membrane sample used). A spectrum generated as the difference between the spectrum in Figure 2B and 2C shows a single spectral line (Figure 2D) with a larger width ($\Delta\nu_{1/2} \sim 100$ Hz) than for the acetylcholine in solution ($\Delta\nu_{1/2} \sim 25$ Hz; Figure 2A). The resonance seen in Figure 2D is seen to occur at 52.35 ± 0.04 ppm, which is 1.6 ppm upfield from the resonance for the acetylcholine in solution (data not shown) or in the solid (Figure 1A). The width of this resonance may reflect heterogeneity in the local environment of the bound substrate, attributable to the two kinetically distinguishable binding sites in the α-subunits of the receptor (*20*), or the presence of three distinct sites for each of the labeled methyls, as a result of the hindered rotation of the quaternary trimethylammonium group within the binding site.

To demonstrate the selectivity of binding of acetylcholine to the receptor agonist binding site observed in Figure 2C, the agonist has been competitively prevented from accessing the binding site using the specific inhibitor of acetylcholine binding, α-bungaratoxin (*20*). The CP-MAS spectrum of receptor-rich membranes treated with α-bungarotoxin (see Material and Methods) in the absence of $N^+(^{13}CH_3)_3$ acetylcholine is shown in Figure 3A. When $N^+(^{13}CH_3)_3$ acetylcholine is added to the membrane at a concentration equivalent to the number of binding sites, no resonance appears in the region between 52 and 54 ppm (Figure 3B) which is where the resonance from $N^+(^{13}CH_3)_3$ acetylcholine should be observed (see Figure 2C for the equivalent spectrum recorded in the absence of α-bungarotoxin).

The significant upfield shift of 1.6 ppm ($p < 0.05$) for the resonance assigned to the $N^+(^{13}CH_3)_3$ acetylcholine bound to the nicotinic acetylcholine receptor demonstrates that $N^+(^{13}CH_3)_3$ experiences a change in electronic environment when bound compared to that in free solution or crystalline solid. Carbon-13 chemical shifts show sensitivity to molecular conformation, local charged environment, and ring currents created by local aromatic groups.

Although not as well understood as proton chemical shifts, carbon-13 chemical shifts show greatest sensitivity to rearrangement of molecular conformation (*21*). However, due to the location of the observed nucleus at the periphery of the molecule, changes in the chemical shift due to conformational distortions are thought to be unlikely.

In the presence of charged residues, the perturbation of the local electron shell has the potential to perturb the observed carbon-13 chemical shift. Such perturbations are thought to have a limited effect (<1.0 ppm) on carbon-13 chemical shift due to the high dielectric of the surrounding environment. The remaining contribution to carbon-13 chemical shifts arises due to the presence of ring currents from local aromatic systems. Simulations of carbon-13 chemical shifts for the $N^+(^{13}CH_3)_3$ quaternary ammonium group have been carried out on the structure of acetylcholine bound to the known crystal structure of the acetylcholinesterase to predict the perturbation expected due to aromatic residues upon binding. This has been done because the binding site of the acetylcholinesterase is rich in aromatic residues and is proposed to have acetylcholine binding site homologous to the nicotinic acetylcholine receptor (*22*). These simulations have yielded a perturbation in carbon-13 chemical shift for the $N^+(^{13}CH_3)_3$ group of −1.6 ppm, in agreement with that observed for acetylcholine bound to the nicotinic acetylcholine receptor.

On the basis of this similarity and the inappropriate nature of the other possible contributions to the $N^+(^{13}CH_3)_3$ chemical shifts, we propose that the acetylcholine binding site on the nicotinic acetylcholine receptor is lined with aromatic residues, as previously suggested on the basis of homology with the acetylcholinesterase (*22*−*25*). Such an observation supports the work of I. Tsigelny and co-workers (*26*), who propose a model of the extracellular domains of the nicotinic acetylcholine receptor containing an acetylcholine binding site rich in aromatic residues. This lends further support to the hypothesis that the interaction between acetylcholine and the nicotinic acetylcholine receptor is mediated primarily through the postively charged quaternary ammonium group interacting with delocalized π systems in the aromatic side chains (*25*).

Residency Time for Acetylcholine Bound to the Nicotinic Acetylcholine Receptor Binding Site. The DDCP experiments carried out on samples which had not been inhibited in agonist binding to the receptor by α-bungarotoxin were analyzed to give the recovery curves shown in Figure 4. All resonances studied (protein backbone, acyl chains, unsaturated groups) as well as those from bound acetylcholine, show a similar exponential increase in spectral intensity with respect to mixing time. Nonlinear least-squares fitting of these curves to the simplified Bloch equation (*27*) for T_{1z} relaxation, assuming the absence of agonist exchange, is

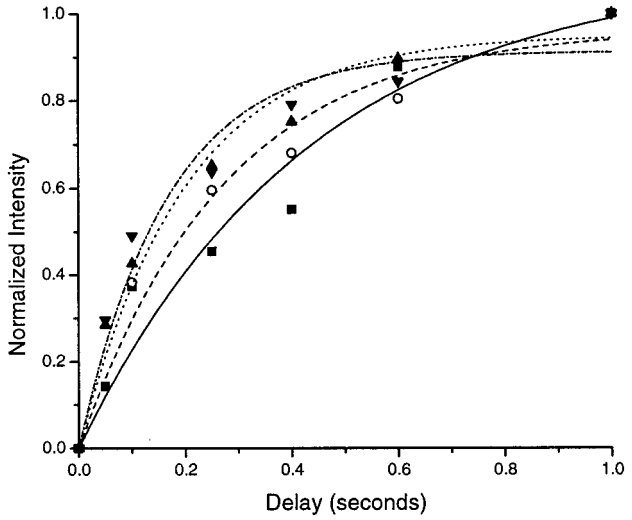

FIGURE 4: Recovery of magnetization for 20 nmol of $N^+(^{13}CH_3)$ acetylcholine added to a sample of acetylcholine receptor membranes (40 nmol binding site) with respect to delay time (τ) during a DDCP experiment. Intensities (I) are plotted with respect to delay time (τ) and normalized with respect to the maximum observed intensity. Protein envelope (■, —, 45−70 ppm), unsaturated resonances (▼, − • −, 128 ppm), $N^+(^{13}CH_3)$ acetylcholine (○, - - -, 52.34 ppm), aliphatic resonances (▲, • • •, 29.00 ppm). Data obtained from DDCP experiments with 1 ms cross polarization time, 1.5 s recycle time, and averaged over 4000 acquisitions. Data processed with 30 Hz linebroadening.

Table 1: Relaxation Data Obtained for Acetylcholine Bound to the Nicotinic Acetylcholine Receptor Membranes[a]

assignment	T_{1z} (s)	χ^2
protein backbone	0.430	0.007
$N^+(^{13}CH_3)_3$ acetylcholine	0.270	0.004
acyl chains	0.190	0.003
unsaturated groups	0.160	0.005

[a] T_{1z} obtained through the least-squares fitting of eq 1 to the DDCP curves shown in Figure 3.

given as

$$M_z = M_o\,(1 - e^{-t/T_{1z}}) \qquad (1)$$

which gives the longitudinal relaxation time (T_{1z}) for the individual spectral components of the membrane (Table 1).

The appearance of magnetization to give a value of T_{1z} of 0.27 s and which can be assigned to the $N^+(^{13}CH_3)_3$ acetylcholine shows a similar rate of appearance to protein ($T_{1z} \sim 0.43$ s) resonances in the spectrum, and is approximately 100 ms slower than that attributed to lipid resonances ($T_{1z} \sim 0.19-0.16$ s). These different relaxation rates would suggest that the appearance of magnetization from bound $N^+(^{13}CH_3)_3$ acetylcholine arises primarily from T_{1z} relaxation while in the protein binding site, rather than from exchange of the bound ligand possessing dephased magnetization with ligand in solution, and which would have therefore retained its magnetization during the initial dephasing period. This result shows that the rate of ligand exchange is long on the T_{1z} time scale (>200 ms), consistent with the previously observed kinetics for agonist binding to the desensitized nicotinic acetylcholine receptor having a K_D of 10^{-9} M and a diffusion-limited on-rate of 10^8 M^{-1} s^{-1} (*28*).

Comparison of the T_{1z} for bound $N^+(^{13}CH_3)_3$ acetylcholine with that of other membrane spectral components (Table 1) indicates that both bound ligand and protein are undergoing a limited range of motions on the nanosecond time scale, which affect T_{1z} relaxation, compared to the lipid resonances where such motions lead to the efficient T_{1z} relaxation. However, the similarity of the T_{1z} for $N^+(^{13}CH_3)_3$ acetylcholine with other membrane protein components, when compared to the T_{1z} measured for such a small molecule when free in solution where T_{1z} relaxation is typically in the order of seconds (*10*), further confirms that the $N^+(^{13}CH_3)_3$ acetylcholine is bound to a membrane environment.

CONCLUSION

It has been demonstrated that CP-MAS NMR can successfully be applied to the study of the $N^+(^{13}CH_3)_3$ acetylcholine bound to the nicotinic acetylcholine receptor. Through the use of the specific inhibitor α-bungarotoxin, it has been possible to assign a resonance that appears at 52.3 ppm to $N^+(^{13}CH_3)_3$ acetylcholine that is bound in a pharmacologically specific manner. The observed 1.6 ppm upfield perturbation in chemical shift for receptor-bound agonist compared with the excess that remains in free solution or the crystalline solid has been assigned to the presence of aromatic residues in the binding site. This provides further evidence that interactions between acetylcholine and the receptor are mediated primarily through cation$-\pi$ bond interactions. Exploiting differing relaxation phenomena between ligands in free solution and those bound to the receptor we have been able to demonstrate that the exchange of $N^+(^{13}CH_3)_3$ acetylcholine is slow on the time scale of the longitudinal relaxation (T_{1z}) in excess of 200 ms.

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