Conformation of acetylcholine bound to the nicotinic acetylcholine receptor

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ABSTRACT We report here the biologically active conformation of acetylcholine when bound to the high-affinity state of the receptor from Torpedo californica. The acetylcholine conformation was determined in the free and bound states by proton NMR two-dimensional nuclear Overhauser effects. In agreement with x-ray crystallographic data, acetylcholine in solution has an extended conformation with an average distance between the acetyl methyl and choline methyl protons of ≈5 Å. When bound to the acetylcholine receptor, acetylcholine adopts a conformation where the acetyl methyl group is close (3.3 Å) to the methyl groups of the choline moieties. This bent conformation places the oxygens adjacent to one another and allows the methyl groups to form an uninterrupted hydrophobic surface over the rest of the acetylcholine molecule. The significant difference between the free- and bound-state conformations implies that structure-activity studies based solely on molecular modeling strategies must be approached with caution.

The physical chemistry of interactions between molecules is a topic of great interest in chemistry, physics, and biology. Interactions that occur leading to recognition in biological molecules are a particularly challenging and important area of research. Studies in this area are difficult, however, because of the large size of at least one molecule involved and because the interactions are usually complex. An understanding of how molecules interact in biological systems may lead to the rational design of molecules that have desired biological functions as well as providing the framework for understanding more complex biological functions such as recognition and assembly.

To understand the interactions of biologically interesting molecules, it is necessary to know the shape of the interaction sites as well as the conformations of the interacting molecules. We report here the biologically active conformation of acetylcholine (AcCho) when bound to the high-affinity (desensitized) state of the acetylcholine receptor (AcChoR) from Torpedo californica. The exact location of the AcCho binding site on the α-subunits of the AcChoR is still unknown, although it has recently been localized to residues 158–216 by several groups using a variety of techniques (1–4). In contrast to the uncertainty in the AcCho binding site, crystal structures are available for AcCho and other agonists that also bind to the AcChoR (5–7). However, we show here that the conformation of AcCho in its receptor-bound state is distinctly different from its conformation in solution and in the crystalline solid state.

MATERIALS AND METHODS

All experiments used purified AcChoR in asolecin vesicles. The receptor was purified from the electroplax organs of T. californica as described (8). We have shown that under the conditions used in this study, AcCho binds to the AcChoR and that selective proton relaxation measurements are very sensitive to this binding. [Under these conditions the ratio of $K_d$ (acetylcholine)/$K_d$ (nicotine) is 0.14.] Conditions (see text) have been determined to distinguish between the contributions from site-specific binding of AcCho to the AcChoR and contributions from nonspecific interactions of AcCho both with the lipid and with other areas of the receptor (8).

All proton NMR spectra were acquired on a 360-MHz Bruker AM series spectrometer with an Aspect 3000 computer. The two-dimensional nuclear Overhauser effect (NOE) data sets were obtained in the phase-sensitive mode (9) and were processed with Dennis Hare’s Fourier transform NMR program. The temperature in all experiments was held at 25°C to within 1°C with the Bruker variable temperature unit by passing heated nitrogen gas through the probe.

The conformations of the AcCho in the free and bound states were determined from proton NMR two-dimensional NOE experiments (10–14). The peak volumes observed for a given mixing time in the two-dimensional NOE experiment are given by

$$V_{nm} = V_0 e^{-Rmn}$$

where $t_m$ is the mixing time in the two-dimensional NOE experiment, and $V_0$ and $V_{nm}$ are the peak volume matrices at times zero and $t_m$, respectively. $R$ is the symmetric relaxation rate matrix describing the magnetic dipolar interactions between the protons in the molecule. Given the volume of the proton peaks in the two-dimensional NOE spectrum at time $t_m$ ($V_{nm}$) and the selective spin-lattice relaxation rate of one proton, the relaxation rate matrix can be determined from Eq. 1 as an eigenvalue problem (9, 15–18). This method for determining the relaxation rate matrix is particularly useful because the effects of indirect magnetization transfer (spin-diffusion) are eliminated (16, 18). The off-diagonal elements of $R$ are called cross-relaxation rates ($\sigma$) and describe the magnetic dipolar interactions between pairs of protons. Although the $\sigma$s depend on both the interproton distance and the molecular motion, the interproton distances can be determined by scaling against the $\sigma$ for a proton pair where the internuclear distance is known (19).

RESULTS AND DISCUSSION

The structure of AcCho and the nomenclature we use is shown on the opposite page. A contour plot of the two-dimensional NOE spectrum of AcCho in $^2$H$_2$O is shown in Fig. 1. The mixing time for this experiment was slightly longer than the selective spin-lattice relaxation time of the choline methyl protons. In this spectrum, the diagonal peaks

Abbreviations: AcCho, acetylcholine; AcChoR, AcCho receptor; NOE, nuclear Overhauser effect.

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are negative and the off-diagonal cross peaks are positive, as expected for a small molecule that tumble rapidly in solution. The presence of a cross peak at a frequency $\omega_1, \omega_2$ shows that the proton at resonance frequency $\omega_2$ is spatially close (<5 Å) to the proton that resonates at frequency $\omega_1$. The larger the volume of the cross peak, the more strongly the protons interact, and therefore the closer the protons are to one another. The peak volumes were measured from this spectrum and the relaxation rate matrix was solved from Eq. 1. The results are shown in the third column of Table 1. The reliability of the two-dimensional NOE analysis in determining the relaxation rates is demonstrated by comparing the diagonal elements ($\rho$) from the two-dimensional NOE rate matrix with the $\rho$ values determined from selective spin-lattice relaxation rate ($R_{1\rho}$) measurements (Table 1, column 2).

The spectrum of AcCho in the presence of AcChoR is shown in Fig. 2. The peaks in this spectrum corresponding to AcCho are labeled and correspond to the labeling on the chemical structure diagram. The additional NMR peaks in this spectrum arise from the lipid (asolectin) in the vesicles and from mobile amino acid side chains on the protein. The amount of asolectin was minimized by eluting the purified receptors from the affinity column without asolectin in the elution buffer (8). The corresponding two-dimensional NOE spectrum of AcCho binding to the AcChoR is shown in Fig. 3. The AcCho (10 mM) was present in large excess compared to the amount of AcChoR (91 μM) present. The relaxation time of the AcCho at this concentration is long compared to the relaxation time of the asolectin (~100 ms) under these conditions, so almost all the lipid has relaxed during the mixing time used in the two-dimensional NOE experiment.

Once again, $\tau_m$ (800 ms) was chosen to be slightly longer than the selective spin-lattice relaxation time of the choline methyl protons (Fig. 3). The AcCho two-dimensional NOE spectrum obtained when AcChoR is present is distinctly different from that for AcCho in $^2$H$_2$O. First, all peaks in the spectrum are negative, which is the expected result for large molecules, or for a ligand bound to a large molecule. Second, there are strong interactions between the acetyl methyl protons and all remaining AcCho protons. These interactions are much weaker in the free AcCho.

Analysis of the spectrum of the bound AcCho is more complicated than for AcCho in $^2$H$_2$O. In the former case, there are contributions to the observed relaxation of the AcCho protons from the free AcCho and from AcCho nonspecifically bound to the lipid and protein in addition to the desired relaxation information from the AcCho specifically bound to the AcChoR binding site. These undesired contributions to the rate matrix from the free AcCho and from the nonspecifically bound AcCho were removed as follows. α-Bungarotoxin was added to the preparation to specifically block binding of the AcCho to the biologically active binding site.

Table 1.Observed relaxation rates (s$^{-1}$) for AcCho from two-dimensional NOE

<table>
<thead>
<tr>
<th>Interaction</th>
<th>In $^2$H$_2$O</th>
<th>With AcChoR/αBTX</th>
<th>Bound to AcChoR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R_{1\rho}$</td>
<td>Two-dimensional NOE</td>
<td>$R_{1\rho}$</td>
</tr>
<tr>
<td>A</td>
<td>0.41 ± 0.01</td>
<td>0.46 ± 0.08</td>
<td>0.51 ± 0.05</td>
</tr>
<tr>
<td>A-B</td>
<td>0.59 ± 0.006</td>
<td>0.02 ± 0.02</td>
<td>0.008 ± 0.009</td>
</tr>
<tr>
<td>A-N(CH$_3$)$_3$</td>
<td>0.05 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>-0.01 ± 0.01</td>
</tr>
<tr>
<td>A-CH$_3$</td>
<td>0.06 ± 0.004</td>
<td>-0.01 ± 0.01</td>
<td>0.10 ± 0.06</td>
</tr>
<tr>
<td>B</td>
<td>0.46 ± 0.01</td>
<td>0.48 ± 0.07</td>
<td>0.64 ± 0.01</td>
</tr>
<tr>
<td>B-N(CH$_3$)$_3$</td>
<td>0.11 ± 0.03</td>
<td>0.07 ± 0.02</td>
<td>-0.02 ± 0.02</td>
</tr>
<tr>
<td>B-CH$_3$</td>
<td>0.004 ± 0.002</td>
<td>-0.02 ± 0.02</td>
<td>-0.02 ± 0.02</td>
</tr>
<tr>
<td>N(CH$_3$)$_3$</td>
<td>0.59 ± 0.02</td>
<td>0.51 ± 0.09</td>
<td>0.71 ± 0.02</td>
</tr>
<tr>
<td>N(CH$_3$)$_3$-CH$_3$</td>
<td>0.005 ± 0.006</td>
<td>-0.02 ± 0.02</td>
<td>-0.02 ± 0.02</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>0.24 ± 0.01</td>
<td>0.29 ± 0.07</td>
<td>0.32 ± 0.01</td>
</tr>
</tbody>
</table>

The errors reported in the rates are the standard deviations of at least three measurements. αBTX, α-bungarotoxin.
site on the AcChoR. The two-dimensional NOE was measured for this system where the specific binding of AcCho is blocked (data not shown), and the resulting relaxation rate matrix (Table 1, column 5) was subtracted from the rate matrix obtained when AcCho was binding to the specific site on the AcChoR (column 7). This difference (19, 20) gives the relaxation rates of the AcCho when bound in the AcChoR site. The relaxation rate matrix for AcCho bound to the lipid was determined by subtracting the rates for AcCho in $^2$H$_2$O from the rates obtained when $\alpha$-bungarotoxin was present in the system.

The interproton distances in AcCho can be calculated from the $\sigma$ values given in Table 1 by scaling against $\sigma$ where the internuclear distance is known. The scaling factor in this system is taken from the B-N(CH$_3$)$_3$ interaction. If the methyl ammonium group is assumed to be in the low-energy staggered conformation, and to undergo a three-site jump about $\tau_1$ in both the free and receptor-bound states, the effective B–N(CH$_3$)$_3$ distance will be unchanged in both the free and bound states. The effective NMR distance ($r'_{ij} = (r^{-3})^{1/2}$) for this interaction is calculated to be 3.0 Å based on geometrical arguments. Given $\sigma_{B-N(CH_3)}$ and $r_{B-N(CH_3)}$, the remaining distances can be calculated from the NMR data by

$$r = \left[ \frac{\sigma_{B-N(CH_3)}}{\sigma} \right]^{1/6} r_{B-N(CH_3)} \quad [2]$$

The following table gives the effective interproton distances in AcCho.

<table>
<thead>
<tr>
<th>Interaction</th>
<th>Crystal structure</th>
<th>In $^2$H$_2$O</th>
<th>Bound to lipid</th>
<th>Bound to AcChoR</th>
</tr>
</thead>
<tbody>
<tr>
<td>A–B</td>
<td>2.6</td>
<td>2.6 ± 0.1</td>
<td>2.3 ± 0.3</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>A–N(CH$_3$)$_3$</td>
<td>3.3</td>
<td>3.5 ± 0.2</td>
<td>3.1 ± 0.4</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>A–CH$_3$</td>
<td>4.3</td>
<td>4.1 ± 0.5</td>
<td>3.0 ± 0.4</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>B–N(CH$_3$)$_3$</td>
<td>3.0</td>
<td>3.0 ± 0.2</td>
<td>3.0 ± 0.4</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>B–CH$_3$</td>
<td>5.2</td>
<td>4.4 ± 0.4</td>
<td>2.8 ± 0.3</td>
<td>3.1 ± 0.3</td>
</tr>
<tr>
<td>N(CH$_3$)$_3$–CH$_3$</td>
<td>5.3</td>
<td>5.5 ± 1.1</td>
<td>3.4 ± 0.6</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

Distances (Å) calculated from $^1$H two-dimensional NOE measurements assuming the effective distance between a B proton and an N-methyl proton is 3.0 Å in bound and free states.

The resulting interproton distances are shown in Table 2. These distances position the protons relative to one another and thereby place limitations on the allowed values for torsion angles $\tau_2$, $\tau_3$, and $\tau_4$ in the molecule (see AcCho structure). The distance between the A protons and the acetyl methyl protons (A–CH$_3$) sets the allowed values for the torsion angle $\tau_4$. The values of the torsion angles $\tau_2$ and $\tau_3$ are then determined primarily by the B proton to acetyl methyl distance (B–CH$_3$), the A proton to acetylcholine methyl (A–CH$_3$), and choline methyl proton to acetylcholine methyl proton [N(CH$_3$)$_3$–CH$_3$] distances. To determine structures that fit the observed interproton distances, the torsion angles $\tau_2$, $\tau_3$, and $\tau_4$ were rotated by angles between 0° and 360° in 5° steps using the torsion angles present in the crystal structure as the starting angles. Allowed structures are those that fit the observed interproton distances within experimental error.

In the AcCho crystal structure (5), the torsion angles are $\tau_2 = 85°$, $\tau_3 = 193°$, and $\tau_4 = 185°$. To get some idea of the sensitivity of the allowed torsion angles to the errors in the interproton distances, the possible structures for AcCho were determined assuming an error of 0.1 Å in the crystal structure interproton distances. The allowed torsion angles are $\tau_4 = 179° ± 12°$,$^4$ $\tau_3 = 184° ± 36°$, and $\tau_2$ has two possible values of $84° ± 8°$ and $277° ± 9°$.

The allowed torsion angles for the solution-state structure of AcCho cover a wide range of conformational space, primarily because of the large error in the N(CH$_3$)$_3$–CH$_3$ and A–CH$_3$ distances. The interproton distances observed by NMR for the AcCho in $^2$H$_2$O match those observed in the crystal structure well, except for the B–CH$_3$ distance. This can be accounted for by small variations about $\tau_4$. This conformation is in agreement with the gauche conformation previously reported by NMR for AcCho in solution (22, 23).

The allowed conformations for AcCho bound to the AcChoR (and lipid) fall into four groups, each with a limited range.

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4 All errors reported in the text and tables represent simple standard deviations.
of torsion angles and all distinctly different from the possible solution structures. Inspection of the structures representing these groups shows that groups 1 and 3 are similar with regard to the placement of their protons, as are groups 2 and 4. The major differences between members of each pair are that (i) the carbonyl oxygens point in opposite directions and are separated by \( =6 \AA \), and (ii) that the nitrogen to carbonyl oxygen distances are \( =5 \AA \) for groups 1 and 4 and \( =5.3 \AA \) for groups 2 and 3.

This analysis shows that the NMR results indicate distinctly different bound- and solution-state conformations. However, the bound-state structure is not uniquely determined from the NMR data alone. The relative energy of each of the structures was therefore calculated to determine the most likely structures. The energy of each structure was calculated by using the Dreiding potentials on the Biograf 1.34 program (Biodesign, Pasadena, CA) with the potential for the \( \tau_4 \) torsion set to zero. The energy for the \( \tau \) torsion (\textit{ab initio} calculations by Krishnan Raghavachari) was added to this result to give the energy for the conformation. A \( \tau_4 \) torsion angle of \( \pm 90^\circ \) has an energy of 13 kcal/mol (1 cal = 4.18 J). The structures in group 3 have the lowest energies, with the lowest energy structure \( (\tau_2 = 270^\circ, \tau_3 = 118^\circ, \text{and} \tau_2 = 220^\circ) \) 7 kcal/mol higher in energy than the crystal conformation. An energy difference of this magnitude between the crystal and bound conformations can easily be compensated in the bound state by new hydrophobic and hydrogen-bonding interactions between the AcCho and the amino acids in the binding site of the AcChoR.

In summary, there are major conformational differences between the free and bound acetylcholine. The major difference is that the arrangement of the N-C-C-O backbone goes from gauche in solution to nearly trans in the bound state. A transoid conformational preference for the muscarinic receptor has been suggested based on a large body of evidence obtained from binding studies on rigid substrates (24). The conformational preference for the nicotine receptor in these studies was not as clear cut.

The bound-state conformation places the two electronegative oxygens on the same side of the AcCho molecule (Fig. 4), and the hydrophobic acetyl methyl and choline methylene groups then form an uninterrupted hydrophobic surface over the rest of the AcCho molecule. This conformation exists when AcCho is bound to the AcChoR as well as when bound to lipid. The bound state conformation of AcCho may reflect the response to a generally hydrophobic environment with charged groups located in the proper places to interact with the charged nitrogen and the electronegative carbonyl oxygen. Such a similarity between the interaction of AcCho with lipid and the AcChoR binding site has already been conjectured (25). The specificity of the AcChoR is then determined by the ability of ligand molecules to match the AcCho bound conformation with the proper distribution of hydrophobic and hydrophilic interactions.

These results are significant for two reasons. It is the first time that the conformation of a neurotransmitter has been determined for the molecule in its bound state. Second, these results point to some inherent caveats in attempts to rationalize the binding activity of pharmacologically active materials. Methods ranging from comparisons of charge densities of these molecules (26, 27) to comparisons of crystal structures from x-ray crystallography and solution state NMR (6, 28) to complex energy calculations (25, 29) have not been entirely successful in predicting the binding activity of various compounds. The reason may be because structure--activity relationships based on crystal structure data or on the conformations of small molecules in solution do not consider the effect of the binding site on the shape of the ligand. In the binding site, the small molecule experiences an environment that is almost certainly different from that of either the solution or the crystalline environment. The binding-site environment may therefore act as a template that induces the ligand to adopt a conformation that differs from the solution or crystalline conformations. This appears to be the case for acetylcholine, where the bound state is distinctly different from the observed solution and solid-state conformations.

Direct observation of other bound-state ligand conformations by the techniques presented here should greatly facilitate the rational design of molecules that interact strongly.
with proteins and DNA and should also provide a fundamental underpinning for understanding the critical aspects of biological recognition and assembly.