

# Proton magnetic resonance studies of cholinergic ligand binding to the acetylcholine receptor in its membrane environment

(nuclear magnetic resonance/choline/cation binding/binding parameters/carbamylcholine)

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**ABSTRACT** Proton magnetic resonance has been used to monitor binding of choline, a known partial agonist, to acetylcholine receptor-enriched membrane preparations from *Torpedo californica* electroplax. The interaction between choline and receptor led to a broadening of the resonance of the choline methyl groups and this effect was reversed by  $\alpha$ -bungarotoxin, a quasi-irreversible antagonist of the acetylcholine receptor. From the concentration dependence of line broadening the equilibrium dissociation constant for choline was obtained ( $K_d = 190 \pm 65 \mu\text{M}$ ). The temperature dependence of the parameters observed in the choline titrations gave an enthalpy of binding  $\Delta H < 1.5$  kcal/mol and allowed estimates for the dissociation rate constant of the receptor-choline complex ( $k_{\text{diss}} > 1.6 \times 10^3 \text{ S}^{-1}$ ) and the respective activation energy,  $E_a$  ( $k_{\text{diss}} \approx 5.5$  kcal/mol). The association of other ligands with the membrane-bound receptor could also be studied by observing effects of varying concentrations of such ligands on the choline methyl group linewidth at a constant choline concentration.

In recent years the application of NMR methodology to biological systems has yielded a wealth of information regarding structural and functional aspects of enzymes and proteins in solution. This has been achieved mainly by approaches such as direct observation of nuclei of constituent amino acid sidechains of the macromolecules or observation of perturbations of ligand nuclei exchanging between free and bound states under different sets of conditions. In order to extend such methods to the study of membrane-bound macromolecules, the second approach is the more feasible provided that (i) a high concentration of membrane-bound ligand binding sites can be obtained, (ii) a ligand is available that is readily observable by using current NMR instrumentation, and (iii) the ligand binding parameters are suitable for study of the exchange between free and bound states.

The third condition is not fulfilled by agonists such as acetylcholine (AcCho) and carbamylcholine (Carb), because these ligands have too high an affinity for the AcCho receptor (AcChoR) under equilibrium conditions. However, all the necessary conditions are met by using choline as a ligand that binds to membranes highly enriched in the postsynaptic nicotinic AcChoR. Titration of membrane fragments enriched in AcChoR (1-3) with choline, a known partial agonist (4), has allowed determination of the choline dissociation constant from the concentration dependence of the observed linewidth, because nonspecific broadening was determined to be negligible by similar titration of membrane fragments in which the AcChoR agonist binding sites were blocked with  $\alpha$ -bungarotoxin ( $\alpha$ -BuTx). The temperature dependence of the dissociation constant and that of the methyl linewidth at a fixed choline

concentration contained the data necessary for determination of the enthalpy of binding and for estimation of the apparent dissociation rate constant and the respective activation energy. Thus, it is possible to apply a highly informative spectroscopic technique to the study of membrane-bound receptors for elucidation of structural and kinetic properties.

## METHODS AND MATERIALS

*Torpedo californica* membrane fragments were enriched in AcChoR by using sucrose density-gradient centrifugation with a Beckman reorienting-gradient rotor (3). The enriched membranes were then centrifuged at  $100,000 \times g$ , and the pellet was resuspended in 0.01 M sodium phosphate buffer made with 99.7%  $^2\text{H}_2\text{O}$ . After a minimum of 2 hr, the membranes were again pelleted and resuspended in 0.01 M sodium phosphate buffer made with 100%  $^2\text{H}_2\text{O}$  (Bio-Rad). This last procedure was repeated and the preparation was then homogenized twice for 30 sec with a VirTis 23 homogenizer operating at maximal speed. Final concentration of AcChoR, expressed as  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx sites, was typically  $10 \mu\text{M}$ . Stock solutions of Carb were freshly made in 100%  $^2\text{H}_2\text{O}$ . Care was taken to minimize atmospheric contact in order to maintain low levels of protiated water, which would lead to technical difficulties involving the dynamic range of the spectrometer.

$^1\text{H}$  spectra were recorded at 100 MHz by using a Varian XL 100-V15 in the Fourier transform mode. Temperatures were controlled by using a modified Varian variable temperature unit. Sample volumes were between 1.5 and 2 ml in 12-mm tubes, which necessitated the use of Teflon Vortex plugs (Wilmad, Buena, NJ).

AcChoR concentration was determined by  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx binding to AcChoR and subsequent adsorption of the complex to DEAE-paper discs (5).

## RESULTS

**Choline Titration.** When a relatively low concentration of choline ( $200 \mu\text{M}$ ) was added to a suspension of AcChoR-enriched membrane fragments at moderate concentration ( $10 \mu\text{M}$  in  $\alpha$ -BuTx binding sites), the linewidth of the three equivalent choline methyl groups was observed to be substantially broader than that of choline alone in aqueous solution (Fig. 1 upper). At a ratio of choline to AcChoR  $\alpha$ -BuTx binding sites of 10:1,

Abbreviations:  $\alpha$ -BuTx,  $\alpha$ -bungarotoxin; Carb, carbamylcholine; AcChoR, acetylcholine receptor.

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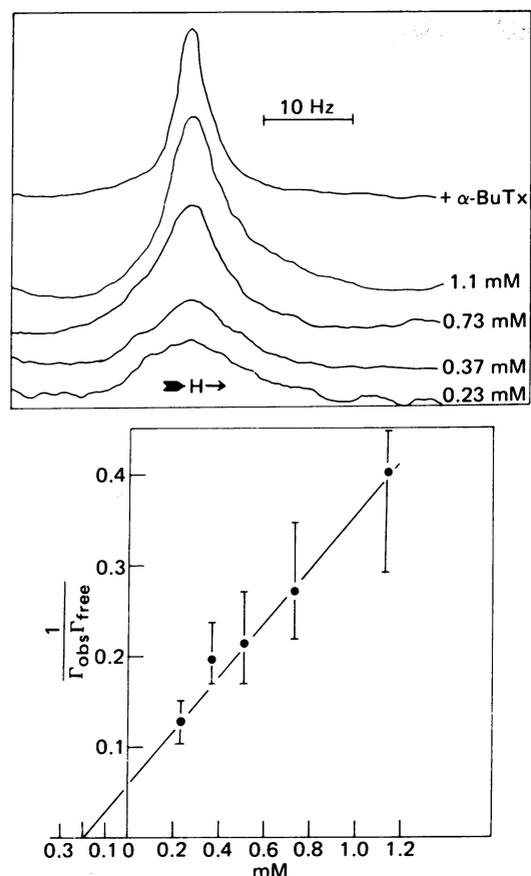


FIG. 1. (Upper) Proton magnetic resonance spectra of choline methyl groups at various concentrations of choline. All spectra were recorded in 10 mM P<sub>i</sub> buffer (<sup>2</sup>H<sub>2</sub>O), pH 7.4, containing membrane fragments with 5 μM AcChoR at 4.6°C. The top spectrum was recorded 20 min after the addition of 10 μM α-BuTx to the above solution in which the choline concentration was 1.1 mM. (Lower) Plot of the spectra in Upper by using Eq. 2; (Γ<sub>obs</sub> - Γ<sub>F</sub>)<sup>-1</sup> versus [choline]. The line was determined by a weighted linear least-squares fit.

a wide (≈15 Hz) line was observed and, as the ratio of choline to AcChoR was increased, the observed linewidth decreased until an apparently constant value was obtained when the choline to AcChoR site ratio was on the order of 100:1. When the same experiment was performed with membrane fragments that had been treated with α-BuTx, a constant and narrow linewidth was observed, independent of the choline concentration. These observations are consistent with the hypothesis that the linebroadening was due to a specific interaction of choline with the AcChoR. No change in chemical shift was observed, and it was considered that this quantity was the same for both bound and free states. In the case of weak binding (see below), the free species is the one observed and, if there is only one bound state, the equation describing the linewidth is:

$$\Gamma_{\text{obs}} - \Gamma_{\text{F}} = \frac{l_{\text{b}}/l_{\text{o}}}{\pi(T_{2\text{b}} + t_{\text{b}})}, \quad [1]$$

where Γ<sub>obs</sub> is the width of the observed lorentzian line at half its height, Γ<sub>F</sub> is the width of the line under conditions in which the specific broadening is abolished,  $l_{\text{b}}/l_{\text{o}}$  is the fraction of the ligand bound (with free ligand in great excess over bound), and  $T_{2\text{b}}$  and  $t_{\text{b}}$  are the spin-spin relaxation time and average residence time of the bound ligand, respectively (6). At a given temperature,  $T_{2\text{b}}$  and  $t_{\text{b}}$  are constant, and the observed broadening is solely a reflection of the fraction of the ligand bound. Thus, the dissociation constant for the ligand may be

obtained by plotting (Γ<sub>obs</sub> - Γ<sub>F</sub>)<sup>-1</sup> versus total ligand concentration,  $l_{\text{o}}$  (Fig. 1 lower), because expressing  $l_{\text{b}}/l_{\text{o}}$  in terms of  $l_{\text{o}}$  and  $K_{\text{d}}$  results in:

$$\frac{1}{\Gamma_{\text{obs}} - \Gamma_{\text{F}}} = \frac{\pi(T_{2\text{b}} + t_{\text{b}})}{r_{\text{o}}} (l_{\text{o}} + K_{\text{d}}). \quad [2]$$

Here,  $r_{\text{o}}$  is the total concentration of binding sites and  $K_{\text{d}}$  is the apparent dissociation constant of the complex.

Several titrations of choline-receptor association at different temperatures were conducted to determine the thermodynamic parameters of the interaction between choline and the AcChoR. For the equilibrium constant little or no temperature dependence was found within our limits of error (Table 1), and it was concluded that Δ*H* for this binding was small.

The temperature dependence of the slopes (S) of the semireciprocal plots, as in Fig. 1 lower, also contains important information:

$$S = \frac{\pi}{r_{\text{o}}} (T_{2\text{b}} + t_{\text{b}}) \quad [3]$$

The two parameters,  $T_{2\text{b}}$  and  $t_{\text{b}}$ , are expected to have opposite temperature dependences (7, 8). We observed a decrease in slope with increasing temperature (see Table 1) and an increase in the observed linewidth at low saturation of the AcChoR with choline. This indicates (see Discussion) that  $t_{\text{b}} > T_{2\text{b}}$ . Because  $t_{\text{b}}$ , the lifetime of the complex, is equal to  $1/k_{\text{diss}}$ , where  $k_{\text{diss}}$  is the (apparent) dissociation rate constant of the complex, one estimates from the data in Table 1 that  $k_{\text{diss}} \geq 1.6 \times 10^3 \text{ S}^{-1}$  at 18°C.

The temperature dependence of the slope (Eq. 3) yields the activation energy for  $k_{\text{diss}}$  with  $E_{\text{a}}(k_{\text{diss}}) = 5 \pm 2 \text{ kcal/mol}$ . A statistically independent estimate of  $E_{\text{a}}(k_{\text{diss}})$  is obtained from an Arrhenius plot of  $\log(\Gamma_{\text{obs}} - \Gamma_{\text{F}})$  at a fixed choline concentration versus  $1/T$  (see Fig. 2). Because  $K_{\text{d}}$  is essentially temperature independent in the temperature range studied (see above and Table 1), the observed slope in Fig. 2 reflects the activation energy of the process characterized by  $t_{\text{b}}$ , which in this case was found to be 5.5 kcal/mol. Both values for  $E_{\text{a}}$  are in good agreement.

**Competition Studies.** Competition studies with radiolabeled ligands have shown that different agonists apparently bind to the same site of the AcChoR from *Torpedo* (9, 10) or *Electrophorus* (11) membranes. Therefore, addition of the agonist Carb should reduce line broadening due to its interaction with the binding site of the ligand being observed (choline). Because Carb affinity for the receptor is much higher than that of choline (10, 12–14), a low concentration of Carb would be undetected in the NMR spectrum but would be expected to narrow the choline signal substantially. This behavior was observed, and a complete titration with Carb at fixed choline concentration (Fig. 3) resulted in an apparent  $K_{\text{d}}$  for Carb of  $3.5 \pm 0.1 \mu\text{M}$  when the data were plotted by using Eq. 2. This value must be corrected for the effect of choline competition by using the

Table 1. Equilibrium dissociation constants and maximum dissociation rates for the choline-AcChoR complex at various temperatures

T, °C	K <sub>d</sub> , μM	(T <sub>2b</sub> + t <sub>b</sub> ), sec
4.6	195 ± 30	9.1 ± 1.4 × 10 <sup>-4</sup>
10.5	220 ± 35	1.05 ± 0.17 × 10 <sup>-3</sup>
18.0	300 ± 70	7.9 ± 1.8 × 10 <sup>-4</sup>
26.0	150 ± 20	4.0 ± 0.5 × 10 <sup>-4</sup>
33.5	100 ± 50	4.3 ± 2.1 × 10 <sup>-4</sup>
Average value	190 ± 65	

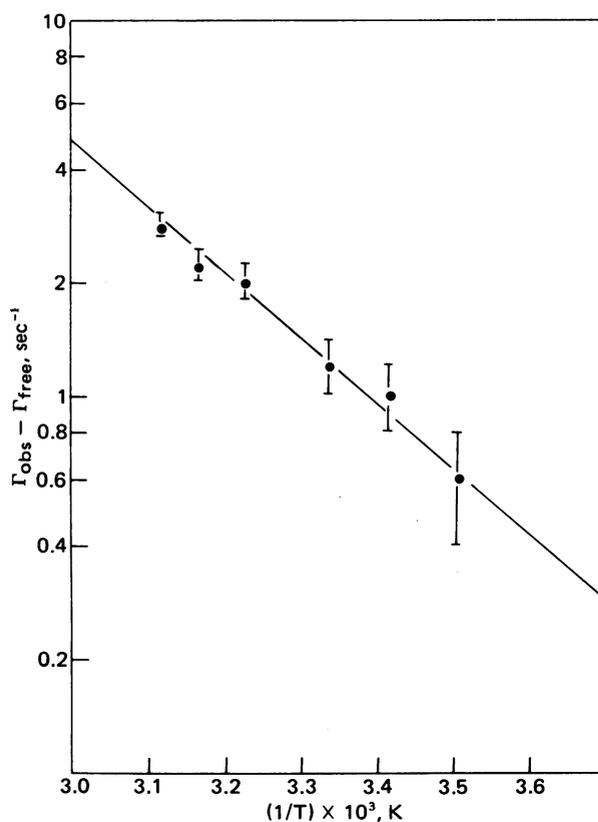


FIG. 2. Arrhenius plot constructed by using data from proton magnetic resonance spectra of a mixture of 0.7 mM choline and AcChoR-enriched membrane fragments ( $^{125}\text{I}$ -labeled  $\alpha$ -BuTx site concentration = 22  $\mu\text{M}$ ) recorded at various temperatures. The plot is of  $\log(\Gamma_{\text{obs}} - \Gamma_{\text{F}})$  versus  $1/T$  and yields  $E_a = 5.5$  kcal/mol.

formula  $K_d = K_{\text{app}} [1 + S_o/K_o]^{-1}$ , where  $K_d$  is the true dissociation constant,  $K_{\text{app}}$  is the apparent dissociation constant measured,  $S_o$  is the concentration of the competing ligand (choline in this case), and  $K_o$  is the dissociation constant of this competing ligand. The corrected  $K_d$  for Carb was  $0.5 \pm 0.1$   $\mu\text{M}$ .

It has been shown that monovalent cations decrease the second-order rate constant for  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx binding to (15) and the affinity of the fluorescent antagonist 1,10-bis(3-aminopyridinium)-decane diiodide for (16) solubilized purified AcChoR. Both of these studies showed that monovalent cations have apparent binding constants of 5 mM. On the other hand sodium chloride, in the 5–50 mM range, had little or no effect on the binding of the agonist AcCho to solubilized purified AcChoR (T. Moody and M. A. Raftery, unpublished results). By using the same methods employed for Carb, we obtained a  $K_i$  (apparent) for NaCl of  $260 \pm 10$  mM, indicating a much weaker effect of salt on the binding of the partial agonist choline than was observed on the binding of antagonist (see above).

The dissociation constant for choline was also determined by inhibition of the kinetics of  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx–AcChoR association (12) in buffer of composition identical to that used in the NMR experiments (i.e., containing  $^2\text{H}_2\text{O}$ ). The value obtained ( $120 \pm 50$   $\mu\text{M}$ ) was in good agreement with that obtained from the NMR data [ $K_d$  (average) =  $190 \pm 65$   $\mu\text{M}$ ]. The same experiment performed with the buffer made in protiated water resulted in essentially the same  $K_d$ .

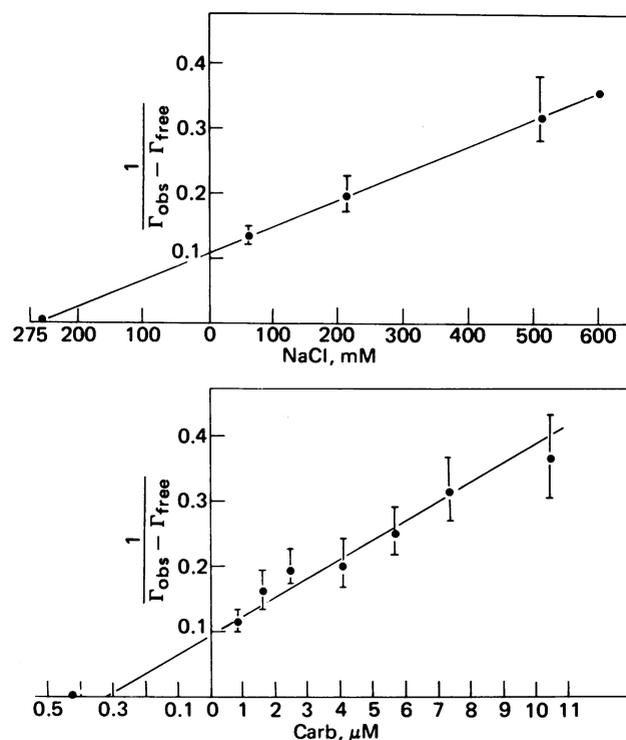


FIG. 3. (Lower) Titration of choline–AcChoR complex with Carb. The experiment monitors the variation of the choline linewidth with Carb concentration. The mixture contained AcChoR-enriched membrane fragments (20  $\mu\text{M}$   $^{125}\text{I}$ -labeled  $\alpha$ -BuTx sites) and 0.9 mM choline in 10 mM  $\text{P}_i$  buffer ( $^2\text{H}_2\text{O}$ ), pH 7.4, at 15°C. The plot shows  $(\Gamma_{\text{obs}} - \Gamma_{\text{F}})^{-1}$  versus [Carb]. The line is a weighted linear least-squares fit, giving an apparent  $K_d$  of  $3.5 \pm 0.1$   $\mu\text{M}$ . (Upper) Titration of choline–AcChoR complex with NaCl. The conditions are as in Lower, with  $(\Gamma_{\text{obs}} - \Gamma_{\text{F}})^{-1}$  versus [NaCl] plotted. The line is a weighted least-squares fit, giving an apparent  $K_d$  of  $260 \pm 10$  mM.

## DISCUSSION

The studies we describe are based on observation of a perturbation in the  $^1\text{H}$ -NMR spectrum of a partial agonist induced by binding interactions with the AcChoR in its native membrane environment. The effect is specific because it can be completely blocked by pretreatment of the membranes with  $\alpha$ -BuTx, which specifically and competitively blocks ligand binding to the AcChoR (17). The binding of  $\alpha$ -BuTx should not affect any linebroadening due to nonspecific interactions, and the data for  $\Gamma_{\text{F}}$  therefore reflect all NMR linebroadening except that due to specific ligand binding at the AcChoR agonist binding site.

The data were analyzed according to Eq. 1, which is a special case of a more general formula derived by Swift and Connick (6) from modified Bloch equations containing terms for chemical exchange (18). The main assumptions leading to Eq. 1 are (i) there are only two kinds of environment for the nucleus (i.e., bound and free), (ii) the free nuclei are always in excess over the bound species and are therefore the ones observed, and (iii) there is no change in chemical shift due to binding.

Assumptions ii and iii were experimentally fulfilled in that we did not observe a chemical shift difference between bound and free ligand, and the concentrations of ligand binding sites used were  $R_o \approx 10$   $\mu\text{M} \ll K_d \approx 200$   $\mu\text{M}$ , so that at all ligand concentrations only a small fraction of the total ligand present was bound. Assumption i was made for the sake of mathematical simplicity because the original formulae of Swift and Connick are complex and would not allow unambiguous determination of the many parameters they contain.

Plotting the observed half-width of the resonances according to Eq. 2 directly yielded the equilibrium constant,  $K_d$ . The value of  $190 \pm 65 \mu\text{M}$  for choline is in agreement with that obtained from inhibition of the kinetics of  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx binding in the same medium ( $120 \pm 50 \mu\text{M}$ ). These values are more than 2 times higher than those determined in 4 mM protiated buffer containing calcium, where we found a value of  $51 \pm 4 \mu\text{M}$  by  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx inhibition (12) and about  $25 \mu\text{M}$  by using the fluorescence probe ethidium (13, 14). An analogous effect was observed for Carb, for which we found  $K_i = 0.5 \pm 0.1 \mu\text{M}$  from competition with choline in our NMR experiments in deuterated, calcium-free buffer compared to  $0.33 \pm 0.05 \mu\text{M}$  by using radiolabeled Carb in calcium-free protiated buffer (M. Schimerlik and M. A. Raftery, unpublished results). On the other hand, values of 0.05–0.12  $\mu\text{M}$  were determined for the  $K_d$  in calcium-containing buffers by using various experimental approaches (13, 14, 17, 19).

The finding of similar equilibrium constants for choline by inhibition of  $^{125}\text{I}$ -labeled  $\alpha$ -BuTx binding kinetics in protiated and deuterated buffers seems to indicate that the lack of calcium is primarily responsible for the high  $K_d$  values. This conclusion is in agreement with the observation of Cohen *et al.* (20) that calcium increases the affinity of the membrane-bound AcChoR for cholinergic ligands. Choline titrations at different temperatures showed essentially no temperature dependence of the equilibrium constant, and the enthalpy of binding was estimated to be  $>1.5 \text{ kcal/mol}$ . Therefore, binding of choline to AcChoR is mainly entropy driven [ $\Delta S \approx 20 \text{ J}/(\text{mol}\cdot\text{K})$ ]. The increased entropy of the complex (compared to the free components) could be due to higher internal flexibility in the complex or a decreased exposure of hydrophobic surfaces of the complex to the solvent. A ligand-induced conformational change of the AcChoR provides a plausible explanation for both cases. There is indeed independent evidence for such an (choline) induced conformational transition of the AcChoR (14, 17, 21). We are not aware of other determinations of the change in enthalpy accompanying agonist binding to the membrane-bound AcChoR. With solubilized purified AcChoR from *Electrophorus*, a considerable positive change in enthalpy was observed upon agonist binding ( $\Delta H \approx 20\text{--}30 \text{ kcal/mol}$ ) which was overcompensated by unusually large positive changes in entropy,  $\Delta S \approx 100\text{--}120 \text{ J}/(\text{mol}\cdot\text{K})$  (22).

Further kinetic results can be inferred from the observed temperature dependence. The denominator of Eq. 1 is the sum of two terms,  $T_{2b}$  and  $t_b$ . Because  $1/t_b$  is the average exchange rate of the ligand–receptor complex, the data obtained from a titration allows us to place a lower limit on the exchange rate. How close this limit is to the actual value depends on the contribution  $T_{2b}$  makes to the denominator.  $T_{2b}$  is not easily evaluated; however, the temperature dependence of the linewidth gives a good indication of the relative sizes of  $T_{2b}$  and  $t_b$  due to their opposite temperature dependences (7, 8). The exchange rate ( $1/t_b$ ) increases with temperature, exposing a larger proportion of the total choline spin population to the bound environment, and this results in a broadening of the observed line as temperature increases.  $T_{2b}$  is expected to have the opposite behavior because the faster molecular motions associated with higher temperature usually result in decreased effectiveness of relaxation processes and thus larger values for  $T_{2b}$  (7, 8). A system in which  $T_{2b}$  was the dominant term in Eq. 1 would be expected to have a decreased linewidth at higher temperature. Our observation that the linewidth increased with temperature (Fig. 2) indicates that  $t_b$  is the dominant term, and, therefore, we may place meaningful lower limits on the dissociation rate of the agonist from the receptor complex. The dissociation rates determined (Table 1) are on the order of 2.5

$\times 10^3 \text{ s}^{-1}$  at room temperature. Assuming a simple  $L + R \rightleftharpoons LR$  mechanism, the lower limit of the association rate constant,  $k_{\text{ass}}$ , can be calculated from  $K_d$  and  $k_{\text{diss}}$  as  $k_{\text{ass}} = k_{\text{diss}}/K_d = 1.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . Values in this range have been observed in stopped-flow experiments for binding of a few agonists to membrane-bound (21, 23) or solubilized purified (24) *Torpedo* AcChoR as well as with *Electrophorus* cells in electrophysiological measurements (25), and they are on the threshold of being diffusion controlled. The activation energy associated with  $k_{\text{ass}}$  is estimated as  $E_a(k_{\text{ass}}) = E_a(k_{\text{diss}}) - \Delta H \approx 5 \text{ kcal/mol}$ , a value also somewhat high for a diffusion-controlled step.

Use of a simple binding mechanism has been widely discussed (4) for choline, a partial agonist; i.e.,



where formation of open channels,  $LR^*$ , is considered to be much less frequent than for full agonists, such as AcCho. Such a situation is in fact favorable for studies of the binding process, rather than activation of channels. It should be kept in mind, however, that our studies have been conducted under equilibrium conditions. We have shown (17) that choline, like other agonists, effectively converts the receptor to a state of higher ligand affinity considered to represent an *in vitro* desensitized—i.e., closed-channel—conformation (26).

There is a possibility that more than one class of agonist binding site exists. The NMR data seem to reflect only a single class of sites; however, sites of significantly higher affinity would be saturated to such an extent as to make a negligible contribution to the observed linewidth. Sites of much lower affinity (14) would escape detection of the transverse relaxation time,  $T_{2b}$ , because the choline methyl protons bound to these sites is similar to that for free choline, as is expected for very loose complexes.

In conclusion, NMR has been shown to be a promising experimental method for investigating agonist binding to the purified AcChoR in its membrane environment. Results that can be compared agree well with those obtained by using other methods, complications due to nonspecific binding effects or probe-induced artifacts can be avoided, and the approach has provided a means of estimating parameters not previously determined.

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