# MEASURING RELATIVE ACETYLCHOLINE RECEPTOR AGONIST BINDING BY SELECTIVE PROTON NUCLEAR MAGNETIC RESONANCE RELAXATION EXPERIMENTS

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ABSTRACT A method is presented that uses selective proton Nuclear Magnetic Resonance (NMR) relaxation measurements of nicotine in the presence of the acetylcholine receptor to obtain relative binding constants for acetylcholine, carbamylcholine, and muscarine. For receptors from *Torpedo californica* the results show that (*a*) the binding constants are in the order acetylcholine > nicotine > carbamylcholine > muscarine; (*b*) selective NMR measurements provide a rapid and direct method for monitoring both the specific and nonspecific binding of agonists to these receptors and to the lipid; (*c*) $\alpha$-bungarotoxin can be used to distinguish between specific and nonspecific binding to the receptor; (*d*) the receptor—substrate interaction causes a large change in the selective relaxation time of the agonists even at concentrations 100x > that of the receptor. This last observation means that these measurements provide a rapid method to monitor drug binding when only small amounts of receptor are available. Furthermore, the binding strategies presented here may be useful for the NMR determination of the conformation of the ligand in its bound state.

## INTRODUCTION

The nicotinic acetylcholine receptor (AChR) is perhaps the best characterized receptor of a neurotransmitter, (1–18) but there remain many unanswered questions about the molecular level interactions between the receptor and its agonists. In particular, it is important to be able to distinguish between specific and nonspecific agonist binding; to be able to assess the importance of interaction between the ligand and the lipid; to have strategies to determine the conformation of the bound agonist; and finally, to have a simple, rapid, and reliable technique to measure agonist binding constants. These questions are well-suited for Nuclear Magnetic Resonance (NMR) spectroscopic investigation, and this manuscript describes results along these lines.

The method presented here is to monitor ligand binding to large receptor molecules by measuring selective spin-lattice relaxation times ($T_1$) of protons on the ligand. The physical picture is one where many small molecules bind and unbind to the receptor and bring back into solution information about their bound state. This information is then read out by selective $T_1$ measurements on the small molecules. In this paper we show (*a*) that selective $T_1$ measurements are a very sensitive measure of ligand binding to the acetylcholine receptor, (*b*) that specific and nonspecific binding effects can be distinguished, and (*c*) that chemical amplification can be obtained by monitoring the on-off binding of many ligand molecules to a low concentration of receptor molecules.

## MATERIALS AND METHODS

### Treatment of the Data

We have a situation where a ligand, *L*, is binding to a receptor molecule, R, and the total ligand concentration is in large excess compared with the receptor concentration. This means that the total amount of ligand which is in the bound state is small. In this case, the spin-lattice relaxation of the protons on the ligand is described by: (19, 20)

$$\frac{1}{T_{1\,\mathrm{obs}}} - \frac{1}{T_{1\,\mathrm{free}}} = \frac{f}{T_{1\,\mathrm{bound}} + \tau_{\mathrm{bound}}}, \qquad (1)$$

where $T_{1\,\mathrm{obs}}$ is the observed spin-lattice relaxation time, $T_{1\,\mathrm{free}}$ and $T_{1\,\mathrm{bound}}$ are the spin-lattice relaxation times of the free and bound ligand, respectively, and $\tau_{\mathrm{bound}}$ is the lifetime of the bound state. The $T_1$'s can be either nonselective or selective spin-lattice relaxation times. (21, 22) The main difference between the selective and nonselective relaxation rates is the frequency independent term, $\tau_c$ in the selective rate. (23, 24) This means that in the regime of slow motions (large $\tau_c$) the selective relaxation rate will be more sensitive that the nonselective relaxation rate. Consequently, selective relaxation times were used to obtain $1/T_{1\,\mathrm{obs}}$.

Ideally, the value of $T_{1\,\mathrm{free}}$ is the relaxation time observed for the ligand alone in aqueous solution. Biologically relevant systems, however, often exhibit nonspecific binding as well as specific ligand binding. The proper value of $T_{1\,\mathrm{free}}$ is therefore the relaxation time of the ligand observed when the specific binding has been completely blocked. The difference between this $T_{1\,\mathrm{free}}$ and the relaxation measured for the ligand alone in solution is a direct result of the nonspecific ligand binding in the biological system. In

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this study, $T_{1\text{free}}$ was measured in ligand-receptor solution after adding $\alpha$-bungarotoxin to block the specific binding site on the AChR protein.

The fraction of the ligand in the bound state, $f$, is $[RL]/[L]_o$ where $[RL]$ is the concentration of the ligand/receptor complex, and $[L]_o$ is the total ligand concentration. The dissociation constant, $K_D$, for the reaction is $[R][L]/[RL]$, and it follows from Eq. 1 that the spin-lattice relaxation in this system is described by (25)

$$[R]_o T_{1P} = [L]_o(T_{1\text{bound}} + \tau_{\text{bound}}) + K_D[T_{1\text{bound}} + \tau_{\text{bound}}], \quad (2)$$

where $[R]_o$ is the total concentration of receptor binding sites and $T_{1P}$ is defined as $[(1/T_{1\text{obs}}) - (1/T_{1\text{free}})]^{-1}$. A plot of $[R]_o T_{1P}$ versus total ligand concentration, $[L]_o$, has a slope of $(T_{1\text{bound}} + \tau_{\text{bound}})$, and $K_D$ can be determined from the intercept.

Once the $K_D$ and $(T_{1\text{bound}} + \tau_{\text{bound}})$ are known for one ligand, the binding constants of other ligands can be quickly determined by measuring the relaxation rate of the previously characterized ligand as a function of competing ligand concentration. The relaxation behavior of the known ligand as a function of competitive inhibitor concentration is described as

$$[R]_o T_{1P} = [I]_o\left[(T_{1\text{bound}} + \tau_{\text{bound}})\frac{K_D}{K_I}\right] + (T_{1\text{bound}} + \tau_{\text{bound}})[K_D + [L]_o], \quad (3)$$

where $[I]_o$ is the total concentration of the competing ligand and $K_I$ is its dissociation constant. A plot of $[R]_o T_{1P}$ versus $[I]_o$ has a slope of $(T_{1\text{bound}} + \tau_{\text{bound}})$ $(K_D/K_I)$. Since $(T_{1\text{bound}} + \tau_{\text{bound}})$ was previously determined (Eq. 2), the slope of Eq. 3 provides directly the ratio of $(K_D/K_I)$.

Note the relative binding strengths for various ligands can be determined even when $K_D$ or $[R]_o$ are unknown if the relative $[R]_o$ is known from measurement to measurement. The ratios of dissociation constants for three or more compounds can easily be determined from the slope of Eq. 3.

## Freeze-Fracture

Freeze-fracture experiments were obtained by the jet-freeze, double replica technique. (26) Approximately 0.1–0.5 $\mu$l of sample was trapped between two thin copper freeze-fracture planchettes (Balzers; Hudson, NH) to form a 10–50 $\mu$m thick layer. The sample sandwiches were rapidly frozen (>15,000°C/s) in the opposing, high velocity jets of liquid propane at −180°C in a Balzers Cryojet 020 apparatus. The frozen sample sandwiches were transferred to a fracture table and loaded into the vacuum chamber of a Balzers 400 freeze-etch device. The fracture table was opened to separate the copper platelets and to fracture the sample under vacuum ($10^{-8}$ torr) at −170°C. The fracture surfaces were replicated by evaporating a 15 Å platinum layer into the sample at a 45° angle, followed by ~ 150 Å of carbon evaporated at normal incidence to provide support. These replicas were observed using a JEOL 100CX electron microscope with an accelerating voltage of 80 kV. Two samples containing receptor plus a control sample of asolectin were observed by this technique.

## Isolation and Purification of Acetylcholine Receptors and Reconstitution into Vesicles

Acetylcholine receptors were isolated from the electroplax organs of freshly-killed *Torpedo californica* obtained from Pacific Biomarine in Venice, California. They were purified and reconstituted into asolectin vesicles according to the procedure described by Huganir and Racker (27). Slight changes were necessary because the presence of excess free asolectin vesicles interferes with (*a*) the protein determination by the dye binding method of Schaffner and Weissman; (28) (*b*) the $\alpha$-bungarotoxin assay described by Ochoa et al.; (7) and (*c*) it broadens the NMR spectra. The amount of free asolectin, and therefore these undesirable side effects, were minimized by eluting the receptors from the affinity column with carbamylcholine without asolectin in the elution buffer.

*Crude Acetylcholine Receptors.* The electroplax organs were minced and added to an equal volume of pH 7.4 2X homogenization buffer (40 mM $Na_2PO_4$, 20 mM EGTA, 10 mM EDTA, 10 mM dithiothreitol, 20 mM benzamidine, 20 $\mu$g/ml leupeptin, 20 $\mu$g/ml antipain, and 20 U/ml trasylol (aprotinin)). Pepstatin (10 $\mu$g/ml), chymostatin (20 $\mu$g/ml), and phenylmethylsulfonyl fluoride (0.1 mM) were added during the first homogenization (Waring blender using eight low speed pulses of 15 s duration, each one separated by one min intervals). The homogenate was centrifuged for 10 min at 6,500 rpm (6,870 *g*) and poured through several layers of cheese cloth. The pellets were suspended in an equal volume of 1X homogenization buffer (2X buffer diluted), recentrifuged, and the supernatant collected as before. The combined supernatants were centrifuged for 2 to 3 h at 13 K rpm (20,200 *g*). The supernatants were then discarded and the pellets were resuspended by adding 10 ml of the 1X homogenization buffer to each tube. Protein concentrations ($A_{280}$, where an absorbence of 1.0 corresponds to a value of 0.6 mg protein/ml [7]) were determined on these crude membrane preparations, and the samples were stored frozen in liquid nitrogen until needed for further purification.

*Purified Acetylcholine Receptors.* The frozen membranes were thawed and brought to a final protein concentration of 2.5 mg/ml in the pH 7.4 purification buffer (20 mM tris-HCl, 100 mM NaCl, 50 mM KCl, 1 mM EDTA, 2 mM EGTA, 10 U/ml trasylol (aprotinin), 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml antipain, 5 mM 2-mercaptoethanol). To this solution, stirring on ice, was added sodium cholate (previously recrystallized from 70% ethanol (8)) to a final cholate concentration of 1%. This solution was stirred on ice for 30 min and then centrifuged for 30 min at 35 K rpm using the Ti 45 rotor (96,000 *g*). The supernatant was combined with the affinity resin (see following section for preparation of the affinity resin) in a supernatant:resin volume ratio of 20:1 and stirred for 2–3 h at 4°C.

After the resin had settled the excess solution was decanted off and the resin was poured into a column, and the column was eluted with 150 ml of the wash buffer (see below) at a fast rate. The protein was eluted from the resin with 50 ml of wash buffer which was 20 mM in carbamyl choline and collected in 1 ml fractions. Fractions were pooled according to their $A_{280}$, and for NMR samples, the pooled protein was vacuum concentrated down to 1–2 ml total volume. It was then dialyzed against three changes of dialysis buffer (20 mM tris-HCl, pH 7.4, 1 mM EGTA, 5 mM dithiothreitol, and 0.4% cholate). A fourth dialysis was performed against $D_2O$-containing dialysis buffer. For NMR samples, the pooled fractions after affinity chromatography were vacuum concentrated to 1–2 ml and dialyzed against three changes of dialysis buffer (20 nM phosphate, pH 7.4, 1 mM EDTA, 5 mM DTT, and 0.4% cholate), followed by three more dialyses against the same buffer prepared with $D_2O$.

The NMR samples were stable for several days at 23°C, and for longer periods at 4°C. Freezing the asolectin-containing sample destroyed the AChR's ability to bind nicotine. The stability (binding activity) of a preparation was monitored before and after long NMR runs by its selective $T_1$.

*Preparation of the Affinity Resin.* Bromoacetylcholine bromide synthesis and the affinity resin preparation were carried out according to Damle et al. (29), and cholate was purified according to Kagawa and Racker (30).

*Toxin Binding Assays.* Toxin binding assays were performed according to the procedure described by Fong and McNamee. (6) We find, as have Ochoa et al. (7) and others, (31) that binding site determinations in asolectin are not reliable, and we have estimated our $\alpha$-bungarotoxin binding site concentrations based on 8 nmol/mg protein. (7) The effective receptor concentrations for ligand binding may be as much as 20% lower due to the presence of multilamellar structures (See Results and Discussion section).

## Proton NMR Relaxation Rate Measurements

All spin-lattice relaxation rates were measured on an AM series 360 MHz wide-bore spectrometer (Bruker Instruments, Inc., Manning Park, Billerica, MA) with an Aspect 3000 data system. The sample temperature was maintained at 22 ± 1°C by passing cooled or heated nitrogen gas over the sample. Nonselective $T_1$'s were measured with the standard inversion-recovery pulse sequence. The 90° pulse width was usually 9.25 μs. Generally 10 $\tau$ values were taken ranging from 0.2 $T_1$ to 2 $T_1$. (32) Sixteen scans provided excellent signal to noise ratio. Selective $T_1$'s were measured with the inversion-recovery experiment where the desired resonance was inverted with a 10 ms pulse from the decoupler. The reported $T_1$ values are those determined by a three-parameter fit using the Bruker software. The relaxation data were always single-exponential over the entire range of $\tau$ values.

*Nicotine Binding Constant Measurement.* Nicotine was added in increments to solutions that typically contained from 7 to 12 mg/ml of protein in $D_2O$. Selective $T_1$ values were meausred at each nicotine concentration.

*Competitive Ligand Titrations.* The NMR samples were typically 60 to 100 μM in receptor binding sites, and had starting nicotine concentrations of 3 mM. Titrations were performed by repeatedly adding a solution of the competing ligand to the existing NMR sample. The $T_{1\text{free}}$ value was determined as the last step by adding a three to four-fold excess of α-bungarotoxin to the NMR sample. The acetylcholine esterase activity of the protein preparation was inhibited in all titrations by adding diisopropyl fluorophosphate (Aldrich).

# RESULTS AND DISCUSSION

## Characterization of the Asolectin/Acetylcholine Preparation used for NMR Studies

Freeze fracture replicas were used to characterize the general morphology of the receptor preparation. The photographs in Fig. 1 show that the samples contain primarily vesicles ranging from <0.02 to 0.2 μm in diameter. Some large multilamellar vesicles are also present (see Fig. 1 *b*). The protein (the pocked small structures, as confirmed by examining a vesicle preparation without protein) in these multilamellar vesicles (Fig. 1 *b*) is encased within several layers of lipid bilayer. The presence of such structures may partially explain the well-known difficulties of $^{125}$I-labeled α-bungarotoxin binding assays, (7, 31) since a small protein such as α-bungarotoxin (74 amino acids) may not diffuse well through the multilamellar structures to protein entrained on the inner layers. It appears that ~1% of the vesicles are large multilamellar vesicles which may contain up to 20% of the total AChR in the sample.

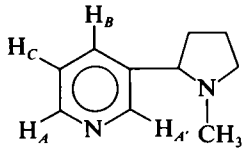
## Characterization of Acetylcholine Esterase Activity of the Receptor Preparation

It is well-known that these receptor preparations contain residual acetylcholine esterase activity because the affinity column purification step also selects for acetylcholine esterase. The esterase activity from this purification scheme was measured by NMR at a low receptor concentration (5 μM in binding sites) by monitoring the intensity of the acetylcholine tetramethylammonium resonance (2.19 ppm) and the intensity of the growing acetate peak (1.93 ppm) as a function of time at 23°C. The sum of these peak intensities remained constant, as expected. The hydrolysis monitored by NMR is a zero order reaction ($k = 0.084 \pm 0.001$ min$^{-1}$).

Adding diisopropyl fluorophosphate to the AChR/asolectin preparation completely inhibited acetylcholine esterase activity as measured by NMR experiments. A small amount of white precipitate formed when the diisopropyl fluorophosphate (1 μl) was added to one to 2 ml of the AChR/asolectin solution, but NMR relaxation measurements show that nicotine binding was unaffected.

## General Characterization of the NMR Spectrum of Nicotine plus the Receptor/Asolectin Preparation

Fig. 2 shows a proton NMR spectrum obtained for 3 mM nicotine in the asolectin/cholate solution containing ~20 μM of receptor binding sites. The agonist, nicotine:




is particularly attractive for competitive ligand binding NMR measurements because it has three main resonances ($H_A$ and $H_{A'}$, $H_B$, and $H_C$, seen from left to right on the insert in Fig. 2) that are shifted several ppm downfield to a region clear of the other resonances. Although well-resolved in $D_2O$ solution, the nicotine $H_A$ and $H_{A'}$ are not resolved in the asolectin/protein preparation used in these experiments. Fig. 3 shows typical selective relaxation data for the $H_B$ proton of nicotine.

The data in Table I illustrate the effect of asolectin, AChR, and competing ligands on the relaxation of the nicotine protons. In $D_2O$, the nicotine $H_B$ relaxation times are between 3 and 4 s. The nicotine $H_B$ relaxation time observed when asolectin vesicles are present at a concentration similar to that present in the titration experiments is considerably shorter than the relaxation time in $D_2O$. This suggests a large amount of nicotine binding to the lipid vesicles, which is not surprising since the positively charged nicotine can interact strongly with the charged lipid head groups. This binding is expected to be nonspecific, and the exchange rate is probably in the fast exchange limit ($T_{1\text{bound}} \gg \tau_{\text{bound}}$).

Large changes occur in the selective relaxation times of the nicotine $H_B$ proton in the presence of the AChR (Table I, third row). Similar changes were observed for the $H_{A,A'}$ and $H_C$ protons (data not shown). These results suggest

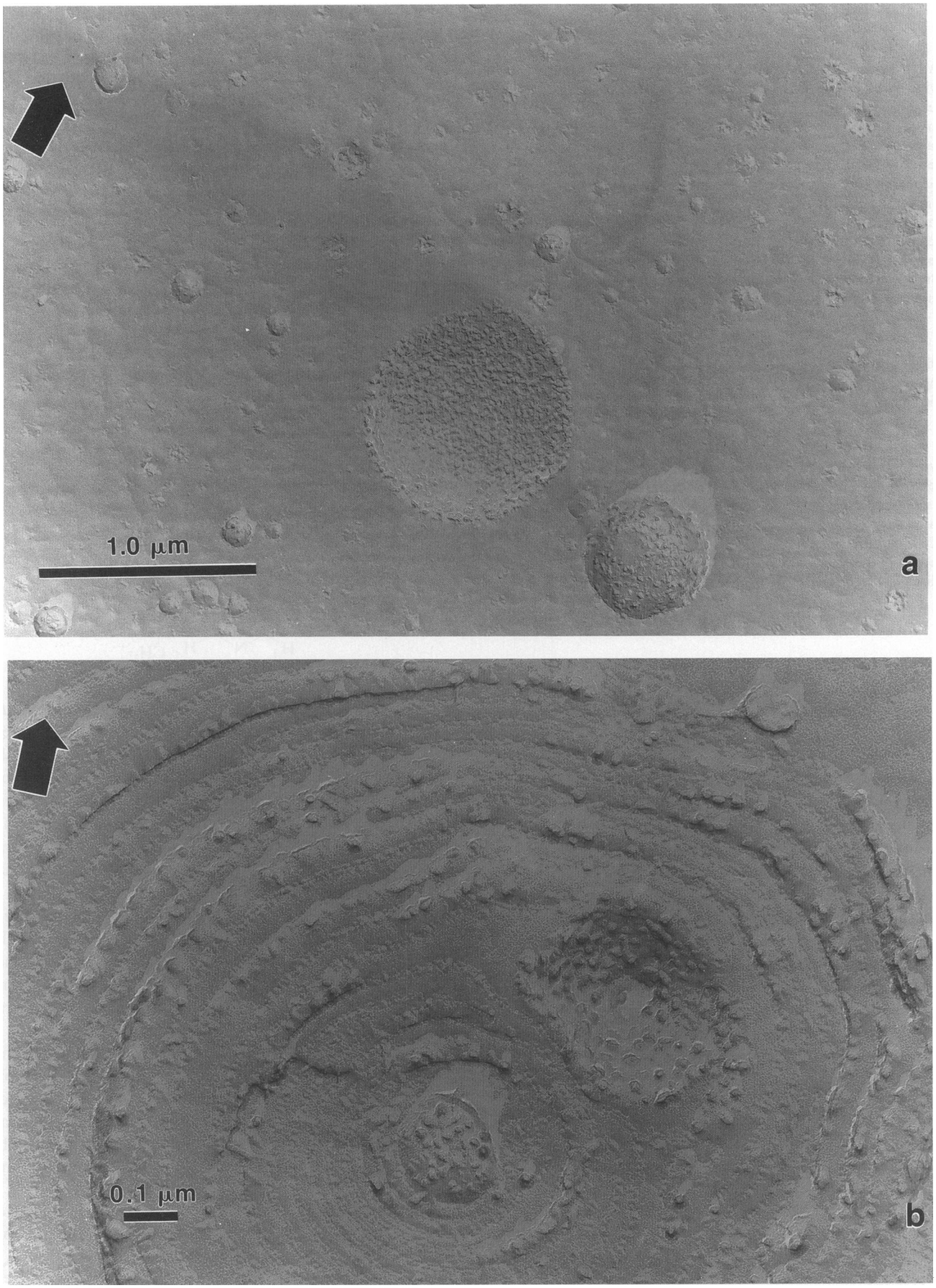



FIGURE 1 Freeze-fracture replicas of a diluted receptor preparation used for NMR studies; (*a*) representative field; (*b*) illustration of multilamellar vesicle in preparation. Arrows indicate platinum shadow direction.

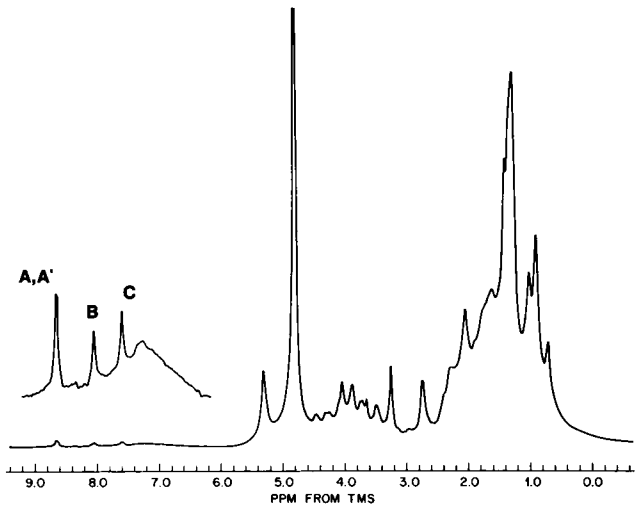



FIGURE 2 Proton NMR spectrum of the AChR/asolectin preparation containing 3 mM nicotine and 10 $\mu$M AChR. The major peaks are labeled on the spectrum.

that the average correlation time of the nicotine molecule changes considerably in the presence of the protein, likely as a result of both specific binding (i.e., to the acetylcholine binding site) and nonspecific binding (i.e., adsorption to or binding with other sites on the protein).

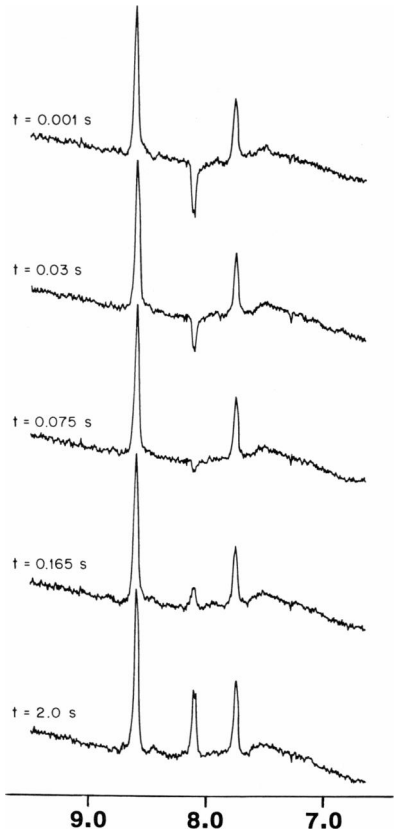



FIGURE 3 Representative selective inversion recovery data for the $H_B$ proton of nicotine in the AChR/asolectin preparation. From left to right, the peaks are assigned to nicotine protons $H_A$ and $H_{A'}$, $H_B$, and $H_C$.

TABLE I
DATA SHOWING EFFECT OF RECEPTOR ON RELAXATION TIMES* (s)

| | $T_{1\text{non-sel}}$ | $T_{1\text{sel}}$ |
|---|---|---|
| Nicotine (10 mM) in $D_2O$ | 3.1 ± 0.3 | 3.6 ± 0.3 |
| Nicotine (10 mM) plus asolectin | 1.97 ± 0.02 | 1.69 ± 0.05 |
| Nicotine (3 mM) plus asolectin/AChR‡ | | 0.36 ± 0.02 |
| Addition of carbamylcholine | | |
| 4.5 mM | | 0.43 ± 0.03 |
| 13 mM | | 0.54 ± 0.01 |
| 19 mM | | 0.70 ± 0.06 |
| Addition of $\alpha$-bungarotoxin | | 1.37 ± 0.03 |

*results are for the nicotine $H_B$ proton.
‡55 $\mu$M in binding sites.

Adding a ligand that competes for binding at the AChR binding site, e.g., carbamylcholine, displaces nicotine from the binding site and thereby changes the observed nicotine relaxation times because the fraction of nicotine bound is changed (Eq. 1, Table I). The observed selective $T_1$ increases because the contribution from $T_{1\text{bound}}$ decreases as the fraction of nicotine bound decreases. Finally, adding $\alpha$-bungarotoxin completely blocks the specific binding of nicotine to the AChR. The measured relaxation time is the $T_{1\text{free}}$ for nicotine in this solution. Note that the selective $T_1$ with $\alpha$-bungarotoxin is similar to the selective $T_1$ when only asolectin is present. The difference between these values is attributed to a small amount of nonspecific binding of nicotine to the AChR.

*Measurement of* $(T_{1\text{bound}} + \tau_{\text{bound}})$ *for Nicotine.* Fig. 4 shows a plot of $[R]_o\ T_{1P}$ versus nicotine concentration for data taken from multiple measurements on two different AChR/asolectin preparations, showing the typical scatter in the data. These data are for the $H_B$ proton of nicotine. The relaxation times of the $H_C$ and $H_{A,A'}$ protons were also examined and found to give similar results (data not shown). The data are linear over a wide range of nicotine concentrations and are reproducible from

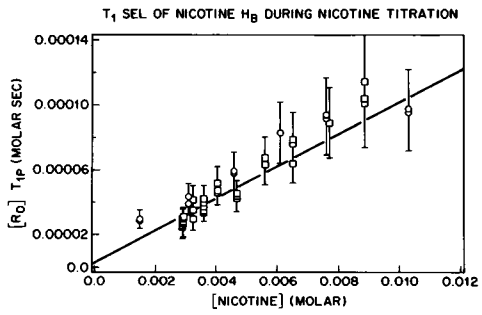



FIGURE 4 Plot of nicotine concentration versus $[R]_o\ T_{1P}$ for multiple measurements on two different AChR/asolectin preparations (*circles and squares*) at 23°C. The $H_B$ proton of nicotine was used for all relaxation measurements. The data show the typical scatter in the selective $T_1$ measurements; error bars were determined by standard methods involving propagation of errors. (41)

preparation to preparation. The averaged results from two titrations are shown in Table II (first row).

*Competitive Titrations for the Receptor Binding Site.* Table I provides illustrative raw data for the competitive titrations; Fig. 5 shows the competitive data plotted for one acetylcholine and for one carbamylcholine titration. Table II summarizes the slopes and intercepts (these are the weighted averages of from 1 to 4 determinations) obtained by plotting the relaxation data according to Eq. 3, as shown in Fig. 5. Since the value of $(T_{1\,\text{bound}} + \tau_{\text{bound}})$ for nicotine is known, these slopes can be used to provide values for $K_D/K_I$ (i.e., $K_{\text{nicotine}}/K_{\text{competing ligand}}$). These values are then combined to give the results summarized in Table III.

The dissociation constants measured here are for the high affinity, or desensitized state, since the receptors were at all times saturated with a large excess of ligand. Table IV lists representative literature values of $K_D$ for acetylcholine and carbamycholine (for which the greatest amount of data are available) for both the low affinity and the high affinity states of AChR from *Torpedo californica*. Also included for comparison are the ratios of $K_{\text{carbamycholine}}/K_{\text{acetylcholine}}$ for both states. Both the absolute values and the ratios of the dissociation constants vary widely from preparation to preparation, again highlighting the difficulties of obtaining absolute values.

## SUMMARY

The results presented here demonstrate that selective $T_1$ measurements are useful for measuring the competitive binding of small molecules to the AChR in its desensitized (high affinity) state. The method exploits the fact that the magnitude of the selective $T_1$ is very sensitive to the zero frequency term, and that this term is affected when the small molecules are temporarily immobilized by binding to the large AChR/asolectin assemblies. The results show that the binding constants are in the order acetylcholine > nicotine > carbamylcholine > muscarine, in agreement with results from other binding assays. This method works particularly well when the ligand has fast dissociation kinetics, which will generally be the case for ligands with dissociation constants of $\approx 10^{-6}$ M and higher. This is recognized as a particularly difficult regime in which to measure $K_D$ by other mthods. (33)

The selective $T_1$ measurements also allow determination

TABLE II
RESULTS FOR NICOTINE TITRATIONS

| Competing ligand | Slope | Intercept |
|---|---|---|
| Nicotine | $(9.9 \pm 0.8) \times 10^{-3}$ | $(2.6 \pm 2.7) \times 10^{-6}$ |
| Acetylcholine | $(6.8 \pm 0.4) \times 10^{-2}$ | $(2.7 \pm 0.1) \times 10^{-5}$ |
| Carbamylcholine | $(0.18 \pm 0.03) \times 10^{-2}$ | $(2.23 \pm 0.1) \times 10^{-5}$ |
| Muscarine | $(0.042 \pm 0.004) \times 10^{-2}$ | $(2.4 \pm 0.1) \times 10^{-5}$ |

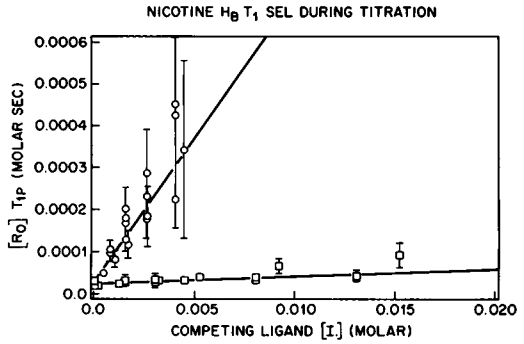



FIGURE 5 Plots of the nicotine $H_B$ relaxation data according to Eq. 3 for competitive titrations with acetylcholine (*circles*) and for carbamylcholine (*squares*). Error bars were determined by standard methods involving propagation of errors. (41)

of the amount of specific binding (i.e., binding to the acetylcholine site on the receptor) versus nonspecific binding (i.e., binding to extraneous sites on the receptor and binding to the lipid vesicles). The results show substantial interactions of acetylcholine and other ligands with the lipid vesicles, but a fairly small amount of nonspecific interactions with the protein.

Because the receptor substrate interaction causes a large change in the selective relaxation times of the small ligands even at concentrations of the ligands that are 100 times greater than the concentration of the receptor binding sites, this method provides a means of chemical amplification; i.e., binding and unbinding of a large amount of ligand to the small amount of receptor continually pumps the solution with molecules that have a "memory" of their bound state. It thereby provides a method to monitor drug binding when only small amounts of the receptor are available. In this fashion, it may be possible to use this method to measure ligand binding to intact cells. It should be possible to extend this technique to other receptors and to other similarly "tethered" biological assemblies.

The minimum molecular mechanism of the AChR involves two states, the low affinity state, and the high affinity state. (6) Even in the absence of agonist, as much

TABLE III
DISSOCIATION CONSTANT RATIOS

| | |
|---|---|
| $\frac{K_{\text{carbamylcholine}}}{K_{\text{acetylcholine}}}$ | 40 ± 7 |
| $\frac{K_{\text{muscarine}}}{K_{\text{acetylcholine}}}$ | 180 ± 30 |
| $\frac{K_{\text{muscarine}}}{K_{\text{carbamylcholine}}}$ | 5 ± 1 |
| $\frac{K_{\text{acetylcholine}}}{K_{\text{nicotine}}}$ | 0.14 ± 0.01 |
| $\frac{K_{\text{carbamylcholine}}}{K_{\text{nicotine}}}$ | 5.6 ± 0.9 |
| $\frac{K_{\text{muscarine}}}{K_{\text{nicotine}}}$ | 26 ± 3 |

TABLE IV
LITERATURE DISSOCIATION CONSTANTS ($\mu$M) FOR ACETYLCHOLINE RECEPTORS FROM TORPEDO

| Preparation | Ligand | Low affinity | | High affinity | | Reference |
|---|---|---|---|---|---|---|
| | | $K_D$ | $K_{D_{carb}}/K_{D_{ach}}$ | $K_D$ | $K_{D_{carb}}/K_{D_{ach}}$ | |
| Electroplax membrane fragments | carb | 20 | | 0.05 | | [11] |
| Membrane fragments | $^3$H-ach | 1.0 | 20 | | | [12] |
| | $^3$H-carb | 20.0 | | | | |
| Membrane fragments (25°C) | ach | 17,500 | 0.52 | 50 | 2.2 | [36] |
| | carb | 9,100 | | 110 | | |
| Triton-solubilized membrane fragments | ach | 2 | | 0.012 | | [37] |
| | nic | 0.5 | | 0.5 | | |
| Membrane fragments | ach | 79.2 | 10.9 | | | [38] |
| | carb | 860 | | | | |
| Membrane fragments | ach | 0.5 | 60 | 0.0014 | 17.9 | [39] |
| | carb | 30 | | 0.025 | | |
| Membrane fragments | ach | | | 0.003 | | [40] |

as 20% of the receptor is thought to be in the high affinity state. However, under the conditions used in this work (large stochiometric excess of acetylcholine or other agonist), essentially all of the receptor is in the high affinity state. Since this method requires a large excess of agonist, it would not work well for the low affinity state unless photolytic (34, 35) or flow methods were worked out to produce a limited amount of active molecule.

The authors wish to thank Drs. R. Huganir and G. Yee for their expert guidance in the preparation of the acetylcholine receptors.

*Received for publication 9 September 1987 and in final form 1 February 1988.*

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