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Molecular Environment of the Phencyclidine Binding Site in the Nicotinic Acetylcholine Receptor Membrane

Andrew L. Palma and Howard H. Wang
Departments of Biology and Chemistry, University of California, Santa Cruz, California 95064

Summary. Phencyclidine is a highly specific noncompetitive inhibitor of the nicotinic acetylcholine receptor. In a novel approach to study this site, a spin-labeled analogue of phencyclidine, 4-phenyl-4-(1-piperidinyl)-2,2,6,6-tetramethylpiperidinoxyl (PPT) was synthesized. The binding of PPT inhibits ^{86}Rb flux (IC_{50} = 6.6 μM), and [^{3}H]phencyclidine binding to both resting and desensitized acetylcholine receptor (IC_{50} = 17 μM and 0.22 μM, respectively). From an indirect Hill plot of the inhibition of [^{3}H]phencyclidine binding by PPT, a Hill coefficient of approximately one was obtained in the presence of carbamylcholine and 0.8 in α-bungarotoxin-treated preparations. Taken together, these results indicate that PPT mimics phencyclidine in its ability to bind to the noncompetitive inhibitor site and is functionally active in blocking ion flux across the acetylcholine receptor channel. Analysis of the electron spin resonance signal of the bound PPT suggests that the environment surrounding the probe within the ion channel is hydrophobic, with a hydrophobicity parameter of 1.09. A dielectric constant for the binding site was estimated to be in the range of 2–3 units.

Key Words phencyclidine · ESR · EPR · nicotinic acetylcholine receptor · spin label · dielectric constant

Introduction

The nicotinic acetylcholine receptor (nAchR) is a transmembrane neuroreceptor protein which functions as an agonist-activated cation channel. In *Torpedo californica* each channel consists of a pentameric complex of four homologous subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$. Electron microscopy of the nicotinic acetylcholine receptor reveals a rosette particle with a diameter of 8–9 nm, containing a 2-nm central pit (Cartaud et al., 1978). X-ray diffraction and electron microscopy data suggest that the receptor has an overall length of 11 nm, extending 5.5 nm beyond the extracellular surface of the membrane and about 1.5 nm into the cytoplasm (Kistler et al., 1982). The existence of membrane-spanning helical regions within subunits has been proposed from analysis of the amino acid sequences (Noda et al., 1982; and *see* Guy & Hucho, 1987, for review). The high degree of homology among the amino acid sequences of the subunits suggests that subunits fold in a similar manner (Noda et al., 1983*a*,*b*) and assemble pseudosymmetrically to form an ion channel.

Two molecules of agonist must bind to the resting nonconducting (*R*) state of the receptor to produce an active, conducting (*A*) state (Neubig, Boyd & Cohen, 1982). Upon biligand complexation of the nAchR with acetylcholine, the lifetime of the *A* state is on the order of milliseconds (Magleby & Stevens, 1972). Prolonged exposure of the acetylcholine receptor to agonist results in another conformational change to a desensitized nonconducting (*D*) state (Katz & Thesleff, 1957). The *D* state is characterized by an increased binding affinity for agonists and a closed ion channel. The rate and extent of desensitization depends on the type and concentration of agonist (Rang & Ritter, 1970; Ochoa, Chattopadhyay & McNamee, 1989).

Noncompetitive inhibitors (NCIs) block the agonist-stimulated cation permeability of the nicotinic acetylcholine receptor without directly interacting with the acetylcholine binding sites. NCIs consist of a heterogeneous group of compounds including the amine local anesthetics, histrionicotoxin (HTx), and phencyclidine (PCP) (Spivak & Albuquerque, 1982). Kinetic analysis of the block of conductance by NCIs (reviewed by Adams, 1981, and Lambert, Durant & Henderson, 1983) and pharmacological studies of the radioligand binding of NCI to the acetylcholine receptor (Heidmann, Oswald & Changeux, 1983; Oswald, Heidmann & Changeux, 1983) has led to the widely accepted view that the majority of NCIs block ion conductance by entering the transiently opened channel and sterically blocking ion passage. However, alternate NCI sites have not been ruled out (Spivak & Albuquerque, 1982).

Phencyclidine (PCP) has been well characterized as a highly specific NCI of the nicotinic acetylcholine receptor (Albuquerque et al., 1980). Radioli-

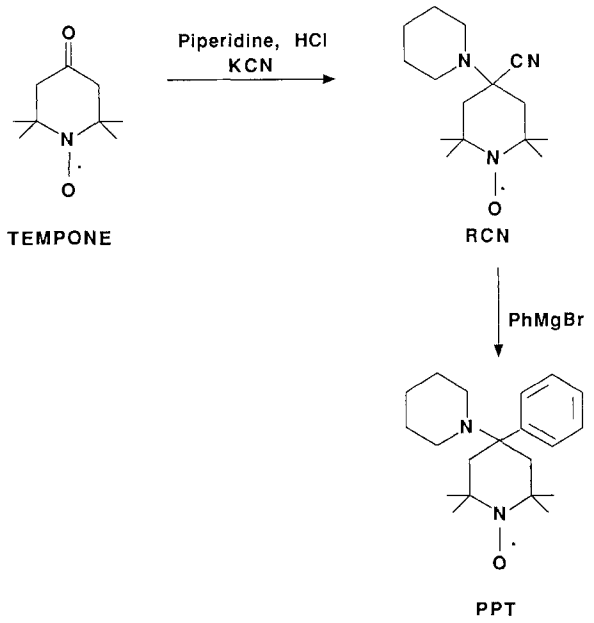

Fig. 1. Reaction scheme for the synthesis of 4-phenyl-4-(1-piperidinyl)-2,2,6,6-tetramethylpiperidinoxyl (PPT)

gand derivatives of PCP bind with high affinity to a single class of NCI sites which are linked allosterically to the agonist binding sites (Aronstam et al., 1981; Heidmann et al., 1983). The stoichiometry of the high affinity sites have been determined to be one per receptor channel (Heidmann et al., 1983; Sobel et al., 1980). In the present study, we utilize these binding characteristics of PCP to synthesize a site-directed spin label capable of specifically monitoring the environment of the NCI binding site within the ion channel.

Materials and Methods

Synthesis of 4-Phenyl-4-(1-Piperidinyl)2,2,6,6-Tetramethylpiperidinooxyl (PPT)

PPT was synthesized according to the procedure of Kalir et al. (1969) for the synthesis of 1-(1-phenylcyclohexyl)piperidine (PCP), with the substitution of 4-oxo-2,2,6,6-tetramethylpiperidinoxyl (TEMPONE) for cyclohexanone. The overall reaction scheme is outlined in Fig. 1. Novel compounds were characterized by their observed atmospheric melting points, infrared spectra, mass spectra, and elemental analysis. Details of the synthesis and results are given in the appendix.

Melting points were obtained on a Thomas Hoover Capillary Melting Point Apparatus and are uncorrected. Infrared spectra were derived from the compounds embedded in KBr pellets with a Nicolet 5-MX FT/IR spectrometer, utilizing the default settings. Mass spectra were recorded on a Finnigan Model 4000 GC/MS. Elemental analyses were performed by either Microtech Laboratories (Skokie, IL) or Atlantic Microlab (Atlantic, GA), as indicated.

Preparation of Native nAchR Membranes

The initial homogenization of the electric organ was performed essentially according to Elliott et al. (1980), with the inclusion of protease inhibitors as described by Saitoh et al. (1980). Native membranes enriched in nicotinic acetylcholine receptors were isolated on discontinuous Percoll gradients by an adaptation of the method of Nagy and Delgado-Escueta (1984), utilizing the density values derived by Lantz et al. (1985). The four-step discontinuous Percoll gradient enriched an acetylcholinesterase fraction, a nonreceptor, α-bungarotoxin binding fraction, and acetylcholine receptor membranes in the first, second, and third steps from the top, respectively. Unless otherwise specified, all manipulations were performed at 4°C. By this procedure, we were able to obtain a native receptor membrane preparation containing 1.2 nmol α-bungarotoxin binding sites/mg protein as determined by the filter disk assay of Damle and Karlin (1978).

$^{86}Rb^+$ Ion Flux

The effect of PCP or PPT on the agonist-dependent ion flux capability of the nicotinic acetylcholine receptor was tested on affinity chromatography purified receptor preparations reconstituted into asolectin liposomes according to Yee, Corley and McNamee (1986). The reconstituted preparation was a gift from Patricia M. Yano and contained a protein concentration of 0.65 mg protein/ml and a mole ratio of lipid to receptor of about 10,000 : 1. The buffer used throughout this experiment consisted of 100 mM NaCl, 10 mM MOPS (pH 7.4), 0.1 mM Na_2EDTA, and 3 mM NaN_3. The $^{86}Rb^+$ influx assay was accomplished by the ion exchange resin technique, according to Lindstrom et al. (1980).

[^{3}H]PCP Binding Assay

Measurement of the binding of [^{3}H]PCP binding to native acetylcholine receptor membranes was performed using the glass fiber filtration method of Eldefrawi et al. (1982). To reduce the nonspecific binding of [^{3}H]PCP to them, the Whatman GF/B glass fiber filters were pretreated with polyethyleneimine as described by Hampton et al. (1982). The amount of [^{3}H]PCP bound to a treated glass fiber filter in the absence of membranes never exceeded 0.3% of the total radioactivity added.

Electron Paramagnetic Resonance (EPR)

Unless otherwise specified, samples were prepared for EPR spectroscopy in 1.5 ml polypropylene microfuge tubes. Native receptor membranes containing 1 mg protein in buffer were added to a buffer solution containing PPT to give a final volume of 1 ml. This mixture was immediately vortexed and centrifuged in a Sorvall SS-34 rotor at 20,000 RPM for 20 min at 4°C. The supernatant was removed by aspiration and the residual pellet placed in a 100 μl calibrated microcapillary tube and sealed.

EPR spectra were recorded with a modified Varian E-3 spectrometer interfaced to a PDP 11/10 computer. The instrumental settings were at a magnetic field set of 3280 gauss, scan range

of 130 G, 100 kHz modulation frequency, 25 mW power, 1 G modulation amplitude, and a 0.1-sec filter time constant. Temperature was maintained by a stream of nitrogen gas regulated at 15 ± 0.5°C. Six-thousand data points across the 130 G scan range were sampled 300 times at each point, digitized, averaged, and stored. Shifting of x-axis registration between spectra could be performed at a resolution of 1 part in 6000; all other spectral manipulations were done on 1000 point files which were averaged from the 6000 point raw data files. The scan time for one spectrum was 6 min. Spectra were generally recorded with signal gains on the order of 10^5 to 10^6.

Protein concentration was estimated by the method of Lowry et al. (1951), with bovine serum albumin as the standard. *Torpedo* lipids were extracted from native acetylcholine receptor membranes by the procedure described by Kolarovic and Fournier (1986) and were made into liposomes by the reverse phase evaporation technique of Szoka and Papahadjopoulos (1978).

Results and Discussion

Effect of PPT on Acetylcholine Receptor Ion Flux

The abilities of PPT and PCP to act as noncompetitive inhibitors (NCIs) of the nicotinic acetylcholine receptor were measured by ion flux assays on reconstituted receptor membranes. Measurable changes were not observed in the ion flux capability following preincubation with either PCP or PPT (concentration ranging from 10^{-7} to 10^{-4} M) for 0 or 30 sec prior to the addition of agonist (*data not shown*). However, preincubation of the drug with the reconstituted receptor membrane for 2 min prior to addition of agonist resulted in PCP and PPT inhibiting ion flux capability in a dose-dependent manner (Fig. 2). PCP and PPT exhibited IC_{50} of 2.1 and 6.8 μM, respectively. These results indicate that PPT does act as an NCI of the acetylcholine receptor, but with about one-third the potency of PCP.

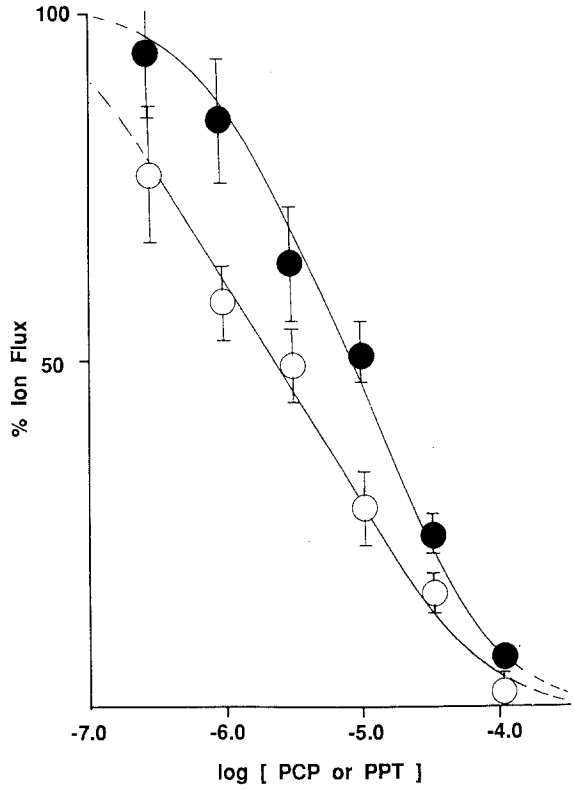

Fig. 2. Log dose-response curve of $^{86}RB^+$ flux across nicotinic acetylcholine receptors. Open circles represent inhibition by PCP; filled circles represent inhibition by PPT

Inhibition of [^{3}H]PCP Binding by PPT

PPT was further characterized as an NCI and an analog of PCP by its ability to compete with [^{3}H]PCP binding to the nAchR. [^{3}H]PCP, in the micromolar range, binds to a single site on the nicotinic acetylcholine receptor with an affinity that is dependent on the state of the receptor (Eldefrawi et al., 1980; Heidmann et al., 1983; Haring & Kloog, 1984). [^{3}H]PCP has a reported K_d of 0.2 to 0.8 μM with desensitized acetylcholine receptor and a K_d of 3 to 5 μM in resting or toxin-treated receptor (Eldefrawi et al., 1980; Heidmann et al., 1983). This binding site is believed to be in the ion channel (Eldefrawi et al., 1980). PPT is able to displace [^{3}H]PCP bound to a desensitized or resting receptor with IC_{50}s of 0.22 (Fig. 3*A*) and 17 μM (Fig. 3*B*), respectively.

Transformation of the data representing 20 to 80% bound [^{3}H]PCP into an indirect Hill plot (Rodbard & Frazier, 1975) yields a slope (n_H) of 0.82 ± 0.05 with an IC_{50} of 0.20 μM in the presence of carbamylcholine. Substitution of α-bungarotoxin for carbamylcholine results in an n_H of 0.66 ± 0.08 with an IC_{50} of 19 μM. Using data representing 25 to 75% of [^{3}H]PCP binding in the indirect Hill plot, as recommended by Cornish-Bowden and Koshland (1973), a n_H of approximately unity for competitive inhibition of [^{3}H]PCP by PPT in the presence of carbamylcholine, and a n_H of 0.8 for toxin-treated acetylcholine receptor (graphs not shown) are obtained. These values are strongly suggestive of direct competition (signified by an n_H of 1) for a homogeneous noncooperative binding site between PPT and [^{3}H]PCP on an agonist-desensitized acetylcholine receptor.

Average values of the IC_{50} were derived from the respective inhibition curves and indirect Hill plots, and then corrected to yield a K_d ($n_H = 1$) or $K_{0.5}$ ($n_H \neq 1$) using the relationship of Cheng and Prusoff (1973). PPT displays a K_d of 0.21 μM for the agonist-desensitized nicotinic acetylcholine recep-

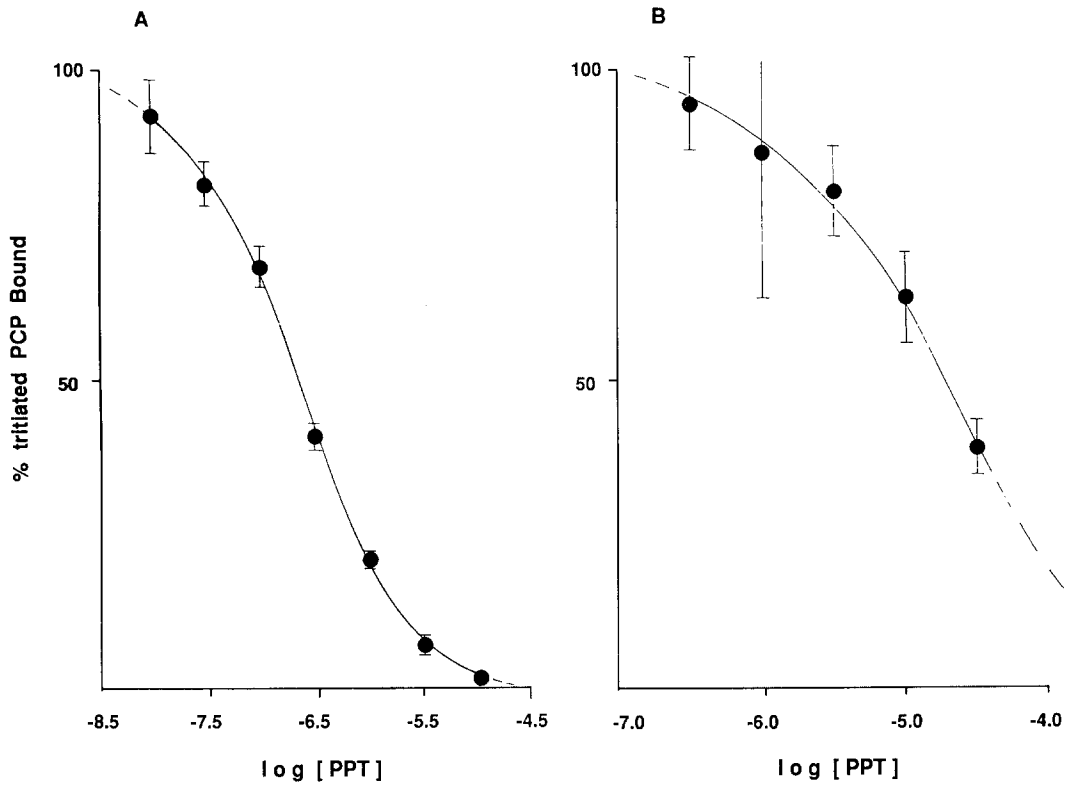

Fig. 3. Inhibition of [^{3}H]PCP binding to nicotinic acetylcholine receptor membranes by (*A*) PPT in the presence of 100 μM carbamylcholine and (*B*) in the presence of 5 μM α-bungarotoxin

tor and a $K_{0.5}$ of 18 μM for the resting toxin-treated receptor.

EPR Spectra of PPT in Membranes

In the presence of extracted lipids from *Torpedo* receptor membranes, PPT shows a weakly immobilized signal (Fig. 4*B*) with an approximate correlation time (τ_c) of 1.73 nsec, estimated by the method of Keith, Bulfield and Snipes (1970). Native acetylcholine receptor membranes in the presence of PPT but without agonist resulted in an EPR spectrum composed of solution and weakly immobilized components (Fig. 4*C*). Addition of agonist introduces an immobilized spectral component (Fig. 4*D*).

Thus, the interaction of PPT with receptor membranes is represented by three distinguishable spectral components. The first is assigned to an aqueous environment, the second is a weakly immobilized component due to association with lipids, and the third, observed in the presence of agonist, is a highly immobilized spectral component representing the interaction between PPT and acetylcholine receptors at the high affinity NCI site.

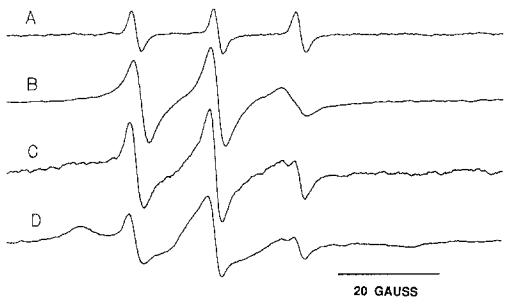

Fig. 4. The spectra were recorded using preparations buffered at pH 7.4. ESR spectra of (*A*) 3 μM PPT in aqueous solution, (*B*) 3 μM PPT in the presence of liposomes of lipids extracted from nAchR membranes, (*C*) 3 μM PPT in the presence of nAchR membrane without carbamylcholine, and (*D*) 3 μM PPT in the presence of nAchR membranes and 100 μM carbamylcholine

The assignment of ligand interaction to a specific site requires the identification of nonspecific interaction (Bennett & Yamamura, 1985; Burt, 1985). Haring and Kloog (1984) defined the nonspecific binding of [^{3}H]PCP in their assay as the binding in the presence of a 100-fold molar excess of unlabeled PCP. We employed a similar stoichiometric ratio of PCP

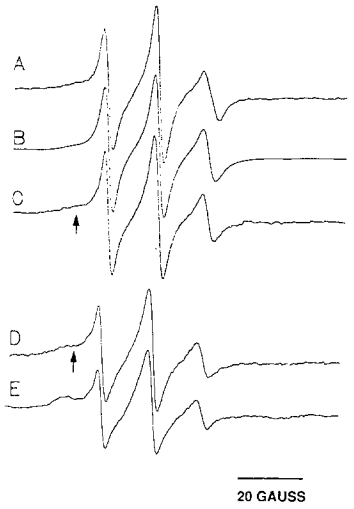

Fig. 5. ESR spectra to illustrate the nonspecific interaction of PPT with nAchR membranes. (All spectra had their solution component removed and their double integral normalized to the same value.) The spectra represent nAchR membrane preincubated with (*A*) 1 mMPCP before adding 10 μM PPT and 100 μM carbamylcholine, pH 7.4; (*B*) 1 mM PCP before adding 10 μM PPT and 100 μM carbamylcholine, pH 9.4; (*C*) 5 mM amantadine HCl before adding 10 μM PPT and 100 μM carbamylcholine, pH 7.4; (*D*) no effectors prior to adding 10 μM PPT, pH 7.4; (*E*) no effectors before adding 10 μM PPT and 100 μM carbamylcholine, pH 7.4. In *A–C* the spectra are identical to those spectra recorded from similar samples, but in the absence of agonist

to PPT to define the initial nonspecific binding EPR spectral component of PPT.

The spectra of the PCP-defined, nonspecific binding component, recorded with the initial bulk aqueous solvent buffered at pH 7.4, are identical in the absence or presence of agonist (Fig. 5*A*) and display narrower line widths than the spectrum from acetylcholine receptors without added effectors (Fig. 5*A*) with an estimated τ_c of 1.43 nsec. This suggests that PPT is more mobile in nAchR membranes in the presence of excess PCP.

At pH 9.4, the nonspecific binding spectra are similar to those recorded at pH 7.4 but contain a larger mobile component, resulting in a τ_c of 1.24 nsec (Fig. 5*B*). The addition of agonist does not alter nonspecific binding, since the excess PCP blocked the agonist-induced binding of PPT. Also the spectra recorded at pH 9.4 lack any apparent trace of a solution component, and the hyperfine splitting is slightly less for these spectra recorded at pH 9.4 than those recorded at pH 7.4. These results indicate that at high pH, a larger fraction of PPT is in the neutral form which favors partitioning into the membrane.

Another method of defining radioligand nonspecific binding utilizes an excess of a chemically dissimilar yet pharmacologically relevant drug to avoid the displacement of some of the radioligand nonspecific binding to tissue, test tubes, and other experimental components, by unlabeled ligand (Bennett & Yamamura, 1985). In the [^{3}H]PCP binding assays of Eldefrawi et al. (1982), the antiviral drug amantadine was employed in this capacity. In our study, the spectra of the nonspecific binding of PPT was also defined in the presence of 5 mM amantadine.

The amantadine spectra, recorded at pH of 7.4 (Fig. 5*C*), are similar to the PCP spectrum. The nonspecific PPT spectrum recorded in the presence of amantadine is not altered in response to agonist, suggesting effective blockage of the high affinity site for PPT. The spectrum is composed of a weakly immobilized signal, and a low amplitude, highly-immobilized signal visible in the low field region (arrow in Figs. 5*C* and *D*) which can be abolished by high concentrations of PCP. Although the pharmacological relevance of this PCP-sensitive residual component is unknown, it is possible that these sites are related to the class of NCI sites postulated by Heidmann et al. (1983) to exist in the vicinity of the annular lipid interface between the nicotinic acetylcholine receptors and the membrane, which may describe the effects of PCP on acetylcholine receptor kinetics not related to channel block (Changeux, Pinset & Ribera, 1986; Papke & Oswald, 1989).

The isotropic hyperfine splitting constant (A_0) of a nitroxide EPR spectrum is highly dependent on the polarity of its immediate surroundings (Griffith, Libertini & Birrell, 1971; Griffith, Dehlinger & Van, 1974). In this study, we compared the polarity of the molecular environment of PPT in solvents, in the presence of liposomes derived from *Torpedo* lipids, and the PCP nonspecific binding component at pH 7.4 and 9.4. Values of the hyperfine splitting for PPT in five different solvents at 15°C are summarized in Table 1. The ESR spectra of PPT in *Torpedo* liposomes and in nAchR membranes (nonspecific component) are all broadened in comparison to the free solution signal exhibited by PPT in solvents. However, since any difference in A_0 due to decreased nitroxide motion in the membrane samples would lead to an overestimation of the true value, the measured values from PPT in membranes were used to estimate the upper limit of the polarity of PPT's environment in those membranes. PPT in *Torpedo* lipids and the PCP nonspecific component, at pH 7.4 and 15°C, are in an environment with a polarity between that of 2-propanol and ethylacetate, while at pH 9.4 and 15°C, the PPT spin labels are in an environment with a polarity between that of ethylacetate and tetradecane. The spectra of PPT

Table 1. Effect of solvent polarity on PPT spectral parameters

Sample	Hyperfine splitting constant A_0(G)	Time constant τ_c(nsec)
H_2O	16.67	—
Ethanol	15.87	—
2-Propanol	15.80	—
Ethylacetate	15.20	—
Tetradecane	15.00	—
Buffer	16.53	—
Torpedo lipids	15.40	1.73
Acetylcholine membranes (nonspecific component)		
pH 7.4	15.47	1.43
pH 9.4	15.13	1.24

in *Torpedo* lipid is indicative of a low polarity environment in the PCP nonspecific binding site, resembling that found in the membrane bilayer. The results show that PPT partitions into an increasingly hydrophobic environment with increasing pH.

Specific Interaction of PPT with Acetylcholine Receptor Membranes

PPT in nicotinic acetylcholine receptor membranes exhibits a strongly immobilized EPR component in the presence of agonist. From the relative strengths of the EPR signals, an increase in the number of spin probes immobilized with agonist stimulation is evident, in agreement with reports of an increase in [^{3}H]PCP specifically binding to the acetylcholine receptor at the high affinity NCI site in the presence of agonist (Eldefrawi et al., 1980; Heidmann et al., 1983; Oswald et al., 1983).

Since both the agonist-induced increase in EPR signal strength and the strong immobilization of PPT by the receptor membranes can be abolished by excess amounts of PCP or amantadine, and competitive binding assays show that PPT and [^{3}H]PCP compete for the same site in the presence of agonist, the strongly immobilized spectral component most likely arises from PPT binding to the acetylcholine receptor high affinity NCI site.

At concentrations of 1 and 3 μM PPT, the spectra produced from PPT's interaction with receptor membranes without effectors (Fig. 6A and C) are essentially identical to the amantadine nonspecific spectra (Fig. 6*B* and *D*). The spectrum produced by 10μM PPT in the absence of agonist (Fig. 6*E*) displays a slightly more broadened component in the low field peak (arrow in Fig. 6*E*) in comparison to the corresponding amantadine nonspecific spectra (Fig. 6*F*).

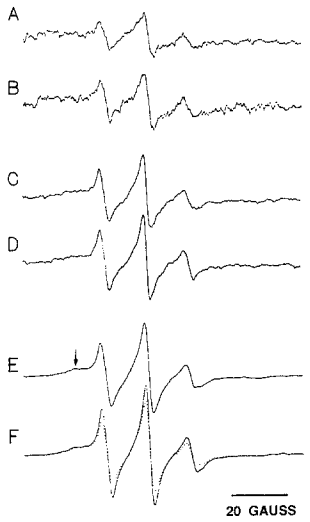

Fig. 6. ESR spectra of PPT associated with nAchR membranes in the absence of agonist. (All spectra had their solution component removed and their double integral normalized to the same value.) 1 μM PPT in the (*A*) absence and (*B*) presence of 5 mM amantadine-HCl; 3 μM PPT in the (*C*) absence and (*D*) presence of 5 mM amantadine-HCl; 10 μM PPT in the (*E*) absence and (*F*) presence of 5 mM amantadine-HCl. The dotted line in *F* is *E*

The agonist-induced spectral component was resolved by subtracting both the aqueous and the nonspecific components. The outer extrema of a strongly immobilized component is easily visible in the initial composite spectra for samples containing agonist. In the resolved spectra, the agonist-induced component resembles a strongly immobilized nitroxide whose separation of the outer extrema are unchanged by the subtraction process. The agonist-induced immobilized component is 67, 62, and 38% of the respective composite spectra at 1, 3, and 10 μM PPT. This result is consistent with the concept of a saturable binding site.

The separation of the outer extrema in the strongly immobilized components is identical for concentrations of 1 and 3 μM PPT, (66.8 $\pm$ 0.3 G) but slightly less for 10 μM PPT (65.2 $\pm$ 0.2 G). This decrease may be due to a rise in mobility of the spin probe (Lassmann et al., 1973), resulting from a local perturbation of the membrane by a high concentration of PPT. Dibucaine, a local anesthetic and an NCI, is known to cause such a disordering of the hydrocarbon core in synaptosomal membranes at concentrations $\geq$10 μM (Ondrias, Stasko & Balgavy, 1987).

There is a direct relationship between the polarity of the nitroxide environment and the limiting isotropic hyperfine splitting, A_{zz} (Lassmann et al., 1973; Griffith et al., 1974). The separation of the outer extrema ($2A_{11}$), in a strongly immobilized spec-

Table 2. Correspondence between isotropic A_0 and limiting A_{zz} values

Solvent	A_0	A_{zz}
Pyridine	15.6	35.01
1-octanol	16.0	35.44
Piperidine	15.4	34.29
Mineral oil	14.92	32.82

trum, approaches the limiting value where $2A_{II} = 2A_{zz}$ (Berliner, 1981).

By using the measured value of 66.8 G for the $2A_{zz}$ value of PPT at the high affinity NCI site, the h parameters described by Lassmann et al. (1973) can be calculated. The h parameter is defined by

$$h = \frac{A_{zz}(H_2O) - A_{zz}(\text{sample})}{A_{zz}(H_2O) - A_{zz}(\text{decaline})},$$

where $2A_{zz}$ is the distance between the outer extrema of the strongly immobilized spectrum. For PPT bound to the acetylcholine receptor, the h parameter was 1.09, which is characteristic of an environment slightly more hydrophobic than decaline.

To determine the dielectric constant for the PPT binding site according to the method of Griffith et al. (1974), we determined the solvent-dependent parameters A_{zz} and A_0 for PPT in mineral oil, piperidine, pyridine, and 1-octanol (Table 2 and Fig. 7). The measured A_{zz} value from the EPR spectrum of PPT in the presence of acetylcholine receptor is 33.4 G which corresponds to an A_0 value of 15.12 G.

The dielectric constant ε is determined from this A_0 value by extrapolating from the correspondence between A_0 values measured from solvents with known dielectric constants (*see* Table 3). By using the value of 15.12 G for receptor-bound PPT, the dielectric constant (ε) of 2.88 was calculated (according to the method of Griffith et al., 1974) assuming no hydrogen bonding is present.

Conclusions

We have shown that PPT inhibits ion flux across reconstituted nicotinic acetylcholine receptor membranes with a potency close to that exhibited by PCP. Since the binding of PPT to the high affinity site in the acetylcholine receptor displaced bound [^{3}H]PCP with a Hill coefficient of about 1.0, PPT apparently competes directly with PCP for the same site. These results indicate that PPT binds to the NCI site and acts as a noncompetitive inhibitor making PPT an excellent probe for the characterization

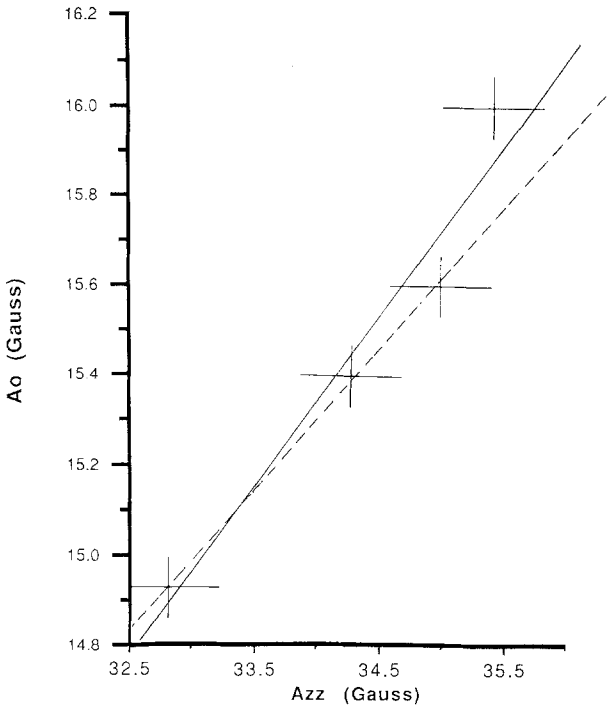

Fig. 7. A plot of A_0 *versus* A_{zz} from values presented in Table 2. The A_{zz} values were determined from the outer extrema (i.e., $2A_{zz}$) of EPR spectra taken from PPT in frozen solvents at liquid nitrogen temperature. The isotropic A_0 values were determined from x-axis crossings of the h_{+1} and h_{-1} peaks (i.e., $2A_0$) of ESR spectra taken from PPT solvents at 25°C. The solid line represents linear regression of all four points. The dashed line is the linear regression from only three points (deleting 1-octanol). However, since the A_{zz} value measured from the sample is 33.4, either line gives close to the same corresponding A_0 value (15.1)

Table 3. Correspondence between ΔA_0 values and dielectric constant of solvents ($\Delta A_0 = 0$ when $\varepsilon = 1$)

Solvents	ΔA_0	$(\varepsilon - 1)/(\varepsilon + 1)$
Acetone	0.54	0.9078
Ethylacetate	0.54	0.7151
Piperidine	0.47	0.7059
Triethylamine	0.20	0.4152
Cyclohexane	0.20	0.3366
Pyridine	0.67	0.8496
Water	2.0	0.9749
Ethanol	1.14	0.9210
Isopropanol	1.07	0.8964
1-octanol	1.07	0.8227

of this site within the receptor. The EPR spectrum of bound PPT indicates that PPT is highly immobilized at the NCI site, and the dielectric environment at this site reflects a highly hydrophobic environment with a hydrophobicity parameter of 1.09. Therefore, by implication, PCP also binds tightly

to a site on the nicotinic acetylcholine receptor, a portion of which has a dielectric constant of approximately 2 to 3 at 15°C. We can conclude that the NCI site has an extremely hydrophobic component and is motionally restrictive.

These findings extend previous observations employing fluorescent NCIs that reflected an environment less polar than water (Grünhagen & Changeux, 1976; Herz, Johnson & Taylor, 1987; and Johnson et al., 1987). Our use of a nitroxide reporter group yields a reliable measurement of the dielectric environment surrounding the bound NCI. We have determined the polarity experienced by the cyclohexyl ring of PCP, but not necessarily those by the phenyl of piperidinyl moieties of PCP. Depending on the location of the cyclohexyl ring within the ion channel, the measurements reflect properties in the core of the membrane (Griffith et al., 1974) or protein (Lassman et al., 1973). An intriguing speculation on the location for PCP's cyclohexyl ring is between the interstices of nicotinic acetylcholine receptor subunits, as suggested for the nonannular sites of the planar cholesterol molecule binding to the receptor (Jones & McNamee, 1988). The presence of such a hydrophobic binding site between the subunits of the pentameric receptor channel is also consistent with our previous finding that uncharged anesthetics are able to reach the channel lumen through a hydrophobic path independent from the channel mouth (Blanton et al., 1988). The location(s) for the phenyl and piperidinyl rings of PCP cannot be determined from our results, but they may reside within the lumen of the ion channel, as has been proposed for the amine and phenyl portions of the noncompetitive inhibitor, QX-222 (Charnet et al., 1990).

These results are direct measurements of the molecular environment in or near the ion channel of the nicotinic acetylcholine receptor and represents an important contribution to our understanding of how channel-blocking drugs bind to their target site. The results emphasize the important contribution of hydrophobic interactions to the activity of drugs upon receptor channels. Such findings suggest a structure of the channel that is more porous, containing hydrophobic spaces between the receptor subunits. We believe drugs with hydrophobic components and anesthetics may bind in such hydrophobic spaces in a saturable manner.

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Appendix

Synthesis of a Spin-Labeled Phencyclidine

Synthesis of 4-oxo-2,2,6,6-tetramethylpiperidinoxyl (TEMPONE) was by oxidation of 2,2,6,6-tetramethyl-4-piperidine monohydrate (Aldrich) as described by Rosen (1974).

Synthesis of 4-Cyano-4-(1-Piperidine)2,2,6,6-Tetramethylpiperidinoxyl (RCN)

1.70 g of piperidine was slowly added to 1.68 ml of HCl (0.02 mol) in 4.00 g of ice-H_2O. The pH was adjusted with dilute HCl to between 3–4. 3.40 g of molten TEMPONE was then added, followed by 1.36 g of KCN in 3.0 ml H_2O. This solution was vigorously stirred for about 2 hr, and then incubated overnight at room temperature. The reaction mixture was extracted with Et_2O (3 × 10 ml), the extracts were combined, dried over anhydrous $MgSO_4$, and evaporated under reduced pressure. The orange residue (4.54 g) was then recrystallized twice from EtOH to give 3.20 g of dark red crystals. (Overall yield = 60%, mp = 97–99°C.)

The IR spectrum (Fig. A1*A*) displays a sharp resonance at 2214 cm^{-1} (arrow, Fig. A1*A*) that correlates well with the characteristic C≡N stretch that appears in the vicinity of 2250 cm^{-1} (Pavia, Lampman & Kriz, 1979). The relatively weak intensity of this absorption, compared to that normally observed for an aliphatic nitrile, is probably due to the dipole moment of the nitroxide being directed in an orientation opposite that of the nitrile.

Anal. $C_{15}H_{26}N_3O$ (performed by Microtech Laboratories)

Expected %:	C 68.14;	H 9.91;	N 15.90.
Found %:	C 67.91;	H 9.65;	N 15.70.

The mass spectrum (*not shown*) was produced by electron-impact ionization at <25 eV. Piperidine nitroxides are notorious for displaying $[M + 1]^+$ ions by addition of a hydrogen from water in the mass spectrometer source (Morrison & Davies, 1970). RCN displayed a base peak (representing the most abundant fragment at 100% relative intensity) at m/e = 192, an $[M + 1]^{+1}$ peak of m/e = 265 (relative intensity ~0.7%), and a $[CM + 1) - 1]^{+1}$ peak of m/e = 264 (relatively intensity ~3.5%). The latter is a characteristic behaviour of aliphatic nitriles, where $[M - 1]^+ > [M]^+$ (in this case, $[(M + 1) - 1]^{+1} > [M + 1]^{+1}$) (McLafferty, 1980).

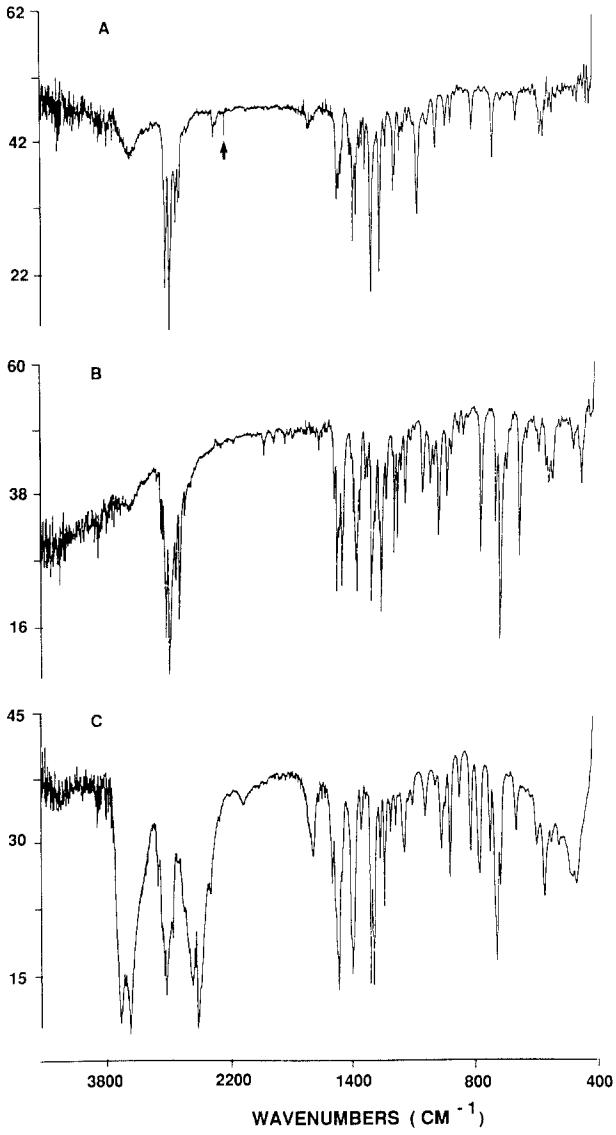

Fig. A1. Infrared spectra in % transmittance for (*A*) 4-cyano-4-(1-piperidinyl)-2,2,6,6-tetramethylpiperidinoxyl, the intermediate compound labeled as *RCN* in Fig. 1; (*B*) PPT free base, the final product shown in Fig. 1; (*C*) PPT hydrochloride precipitated from isopropanol:conc. HCl (5 : 1 vol/vol)

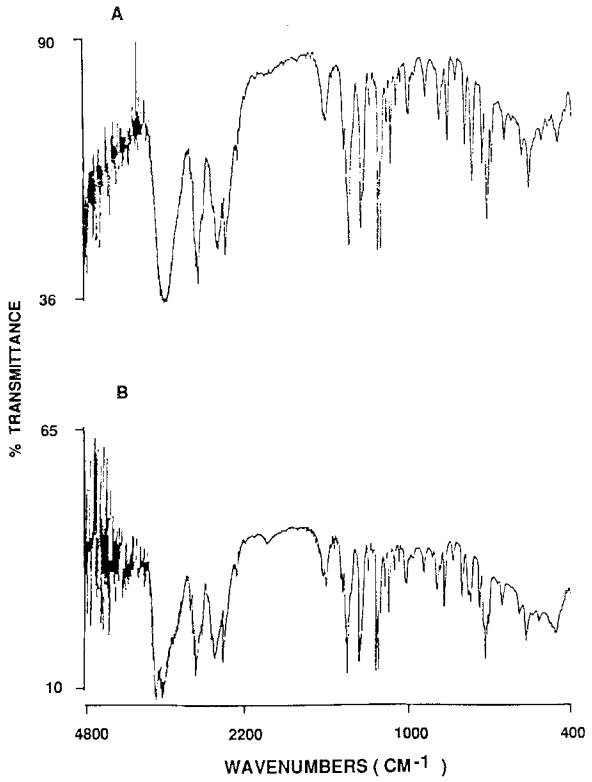

Fig. A2. Infrared spectra of PPT hydrochloride. (*A*) Anhydrous preparation (*see* text). (*B*) The hydrated product of *A*, PPT-HCl · H_2O

Synthesis of 4-Phenyl-4-(1-Piperidinyl)-2,2,6,6-Tetramethylpiperidinoxyl (PPT)

507 mg (1.92 mmol) of RCN was dissolved in 3.5 ml of Et_2O. 650 μl of a 3 M solution of phenylmagnesium bromide in Et_2O (Aldrich) was added by drops, to form a viscous precipitate. The mixture was allowed to stand for 1.5 hr, then poured into an ice-cold saturated NH_4Cl solution. The Et_2O layer was separated and washed with dH_2O. The base was extracted with 1 M HCl, resuspended with concentrated NH_4OH, re-extracted with Et_2O, and dried and evaporated under reduced pressure. The resulting material was recrystallized twice from EtOH to give 101 mg of bright orange needles. Yield-17%, mp = 156–158°C.

The IR spectrum (Fig. A1*B*) displays strong absorption bands at 704 and 768 cm^{-1} (arrows, Fig. A1*B*) which are near the characteristic 690 and 750 cm^{-1} resonances for the monosubstituted aromatic =C—H out-of-plane bending (Pavia et al., 1979). The spectrum also shows the concomitant loss of the C≡N stretch in the vicinity of 2214 cm^{-1}.

Anal. $C_{20}H_{31}N_2O$ (performed by Microtech Laboratories)

Expected %:	C 76.14;	H 9.90;	N 8.88
Found %:	C 76.36;	H 9.97;	N 8.69.

The mass spectrum (*not shown*) was produced by electron impact ionization at <25 eV. The base peak was observed at an m/e = 74, and an $[M + 1]^+$ peak of m/e = 316 (relative intensity ~10%).

Synthesis of 4-Phenyl-4(1-Piperidinyl)-2,2,6,6-Tetramethylpiperidinoxyl Hydrochloride Monohydrate

152.82 mg (0.482 mmol) of PPT base was dissolved in 5 ml Et_2O and 420 μl of 1 N HCl (made from a 5 : 1 (vol/vol) mixture of isopropanol : concentrated HCl subsequently dried over anhydrous Na_2SO_4) was added. 134 mg of an orange-pink powder was recovered. Yield was 86%. Decomposition occurred at 146°C.

Anal. $C_{20}H_{32}N_2OCl \cdot H_2O$ (performed by Atlantic Microlab)

Expected %:	C 64.93;	H 9.26;	N 7.57;	Cl 9.58
Found %:	C 64.80;	H 9.26;	N 7.48;	Cl 9.63.

The IR spectrum of this compound, embedded in KBr (Fig. A1*C*), contains a doublet resonance in the region of 3500 cm^{-1}. This is curious, since this sort of absorbance pattern is generally indicative of an NH_2 stretch, which should not be present. The free base form of PPT apparently does not contain an N-H stretch, which would be necessary to give rise to an $R_2NH_2^+$ moiety in the hydrochloride salt. (Note that the absorbance in the region of 3460 cm^{-1} in Fig. A1*A* and *B*, though possibly indicative of an N-H stretch, is assigned to traces of moisture in the sample preparation. This assignment was based on the variability of the intensity of this absorbance from preparation to preparation.)

This doublet pattern is most likely due to the overlap of O-H and N-H stretches. This explanation is consistent with the presence of a molecule of hydrated water which had been tentatively assigned to the compound's structure, based on the results from the elemental analysis.

Proof of this was afforded by the formation of the hydrochloride salt of PPT employing anhydrous isopropanolic HCl (made by bubbling HCl gas through isopropanol). This compound was of a dull pink color which displays a single IR resonance around 3450 cm^{-1} (Fig. A2*A*). Hydration of the anhydrous PPT hydrochloride salt turned it into a bright orange-pink color and resulted in the same IR spectral pattern as previously observed (*compare* Fig. A2*B* with A1*C*).

The mass spectrum for PPT-HCl · H_2O produced by electron impact ionization, at <25 eV, yielded the same pattern as the free base compound (*not shown*). This is to be expected, as amine salts often decompose upon heating in the mass spectrometer to release the free amine and the acid (McLafferty, 1980). Production of the mass spectrum by chemical ionization with NH_3 (*not shown*) yielded a base peak of m/e = 86, and a peak related to the original molecular structure at m/e = 389 (relative intensity ~2%). Since chemical ionization with NH_3 as the reagent gas initially produces a collision-stabilized complex between its conjugate acid and the molecule $[M(NH_4)]^+$ (Richter & Schwarz, 1978), and assuming that the nitroxide underwent reduction by addition of a hydrogen radical from the reagent gas, the parent-related peak should therefore correlate to $[M + 19]^+$. The resultant mol wt of 370 matches the expected mol wt for a monohydrated molecule of PPT-HCl.