Binding Sites for α-Bungarotoxin and the Noncompetitive Inhibitor Phencyclidine on a Synthetic Peptide Comprising Residues 172–227 of the α-Subunit of the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The binding of the competitive antagonist α-bungarotoxin (α-Btx) and the noncompetitive inhibitor phencyclidine (PCP) to a synthetic peptide comprising residues 172–227 of the α-subunit of the *Torpedo* acetylcholine receptor has been characterized. ^{125}I-α-Btx bound to the 172–227 peptide in a solid-phase assay and was competed by α-Btx ($IC_{50} = 5.0 \times 10^{-8}$ M), *d*-tubocurarine ($IC_{50} = 5.9 \times 10^{-5}$ M), and NaCl ($IC_{50} = 7.9 \times 10^{-2}$ M). In the presence of 0.02% sodium dodecyl sulfate, ^{125}I-α-Btx bound to the 56-residue peptide with a K_D of 3.5 nM, as determined by equilibrium saturation binding studies. Because α-Btx binds to a peptide comprising residues 173–204 with the same affinity and does not bind to a peptide comprising residues 205–227, the competitive antagonist and hence agonist binding site lies between residues 173 and 204. After photoaffinity labeling, [^{3}H]PCP was bound to the 172–227 peptide. [^{3}H]PCP binding was inhibited by chlorpromazine ($IC_{50} = 6.3 \times 10^{-5}$ M), tetracaine ($IC_{50} = 4.2 \times 10^{-6}$ M), and dibucaine ($IC_{50} = 2.7 \times 10^{-4}$ M). Equilibrium saturation binding studies in the presence of 0.02% sodium dodecyl sulfate showed that [^{3}H]PCP bound at two sites, a major site of high affinity with an apparent K_D of 0.4 μM and a minor low-affinity site with an apparent K_D of 4.6 μM. High-affinity binding occurred at a single site on peptide 205–227 ($K_D = 0.27$ μM) and was competed by chlorpromazine but not by α-Btx. α-Btx and chlorpromazine competed the minor binding component on the 172–227 and 173–204 petpides. It is concluded that a high-affinity binding site for PCP is located between residues 205 and 227, which includes the first 18 residues of transmembrane segment M1, and that a low-affinity site is located in the competitive antagonist binding site between residues 173 and 204. These results show that a synthetic peptide comprising residues 172–227 of the α-subunit contains three binding sites, one for α-Btx and two for PCP. Previous studies on the intact receptor indicate high-affinity PCP binding occurs in the receptor channel. Thus, the high-affinity site within residues 205–227 may be exposed or closely approximated to the ion channel of the receptor. The proximity of the agonist binding site to a noncompetitive inhibitor binding site on the linear sequence of the α-subunit suggests that these sites could be tightly coupled and that this region could be involved in gating of the channel.

The nicotinic acetylcholine receptor (AChR)[1] is a transmembrane glycoprotein that has been well characterized physiologically and biochemically (Karlin, 1980; Popot & Changeux, 1984; Hucho, 1986; Karlin et al., 1986; McCarthy et al., 1986; Changeux et al., 1987; Stroud et al., 1990). The purified AChR isolated from *Torpedo californica* electric organ is composed of four subunits present in a stoichiometric ratio of $2\alpha1/\beta1/\gamma/\delta$. This pentameric complex contains the binding sites for agonists [e.g., acetylcholine (ACh) and carbamylcholine], antagonists [e.g., α-bungarotoxin (α-Btx) and *d*-tubocurarine], and noncompetitive inhibitors (NCIs), which block cation flux by binding to sites other than the ACh binding site. In addition, the five subunits are responsible for forming the ion channel. The primary structures of all four subunits of the *Torpedo* receptor, as well as subunits from other species, have been deduced from the sequences of the cloned cDNAs [see Noda et al. (1983)]. The subunits are transmembrane proteins with considerable sequence homology. Hydrophobicity analysis of the primary sequences predicts that each subunit possesses at least four transmembrane helices labeled M1 through M4 (Claudio et al., 1983).

Cholinergic ligands have been used to localize the ACh binding site on the AChR. The snake venom neurotoxin α-Btx has been utilized as a probe for the cholinergic binding site because it binds specifically and with high affinity to the AChR and competitively blocks the depolarizing action of ACh. Studies investigating the binding of α-Btx to isolated α-subunit (Haggerty & Froehner, 1981; Gershoni et al., 1983; Oblas et al., 1983), proteolytic peptide fragments (Tzartos & Changeux, 1983; Wilson et al., 1984; Pedersen et al., 1986), synthetic peptides (Wilson et al., 1985; Mulac-Jericevic & Atassi, 1986; Neumann et al., 1986; Ralston et al., 1987; Wilson & Lentz, 1988; Wilson et al., 1988; Conti-Tronconi et al., 1990), and fusion proteins containing receptor sequences (Barkas et al., 1987; Gershoni, 1987) indicate a major neurotoxin binding site is located between residues 173 and 204 of the α-subunit. Synthetic peptides comprising residues 125–148 (Mulac-Jericevic & Atassi, 1986) and 55–74 (Conti-Tronconi et al., 1990) have also been reported to bind α-Btx,

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; BSA, bovine serum albumin; α-Btx, α-bungarotoxin; CPZ, chlorpromazine; DDF, *p*-(*N*,*N*-dimethylamino)benzenediazonium fluoroborate; MBTA, [4-(*N*-maleimido)benzyl]trimethylammonium iodide; NCI, noncompetitive inhibitor; PB, phosphate buffer; PBS, phosphate-buffered saline; PCP, phencyclidine; SDS, sodium dodecyl sulfate; UV, ultraviolet.

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although not to the extent of sequences containing Cys-192 and Cys-193. A cyanogen bromide fragment comprising residues 179–207 is labeled by affinity alkylating agents such as [4-(*N*-maleimido)benzyl]trimethylammonium iodide (MBTA) (Kao et al., 1984; Dennis et al., 1986) and the cholinergic photoaffinity ligand *p*-(*N*,*N*-dimethylamino)-benzenediazonium fluoroborate (DDF) (Dennis et al., 1986). MBTA labeled Cys-192 and Cys-193 (Kao et al., 1984), and DDF labeled Trp-149, Tyr-190, Cys-192, and Cys-193 (Dennis et al., 1988) and Tyr-93 (Galzi et al., 1990) of the α-subunit. These studies point most strongly to the sequence flanking Cys-192 and Cys-193 of the AChR α-subunit as containing the major binding site for cholinergic ligands, with a possible contribution to the site by other regions of the α-subunit.

NCIs are a heterogeneous group of compounds that includes aminated local anesthetics, histrionicotoxin, quinacrine, phenothiazines, and phencyclidine (PCP). NCIs bind to a high-affinity site distinct from but allosterically coupled to the ACh binding site and when bound block the permeability response of the receptor (Changeux, 1981; Heidmann et al., 1983; Karlin et al., 1986; Changeux & Revah, 1987; Changeux et al., 1987). NCIs could block the function of the AChR by stabilizing the desensitized state, by occluding the ion channel, or by interacting with the domains involved in the opening of the channel. These ligands bind to at least three sites on the AChR: (1) a high-affinity site considered to lie in the channel, (2) several low-affinity sites suggested to lie along the lipid bilayer, and (3) the ACh binding site. When the AChR is in the open channel state in response to agonists, NCI binding to the high-affinity site is enhanced. NCIs by promoting desensitization of the AChR enhance the binding of agonists. Since NCIs label sites on the receptor coupled to the opening/closing of the ion channel, they represent useful probes to identify these functionally important domains and to reveal sites on the transmembrane segments facing the lumen of the ion channel.

Available evidence indicates NCIs bind to a site on transmembrane segment M2 and possibly also M1 suggesting that these segments line the ion channel or are closely approximated to it. Chlorpromazine (CPZ) has been shown to label all four subunits with identical kinetics (Oswald & Changeux, 1981; Heidmann & Changeux, 1986). Triphenylmethylphosphonium (Hucho et al., 1986) and CPZ (Giraudat et al., 1989) label homologous residues in putative membrane-spanning helixes M2. Thus, it was hypothesized that homologous regions of transmembrane segments M2 contribute to the high-affinity NCI site (Giraudat et al., 1987) and that the channel is formed by the M2 segments of all of the subunits (Hucho et al., 1986). When quinacrine azide was bound to the receptor in the open state, a cyanogen bromide fragment containing transmembrane segment M1 of the α-subunit was labeled (Di Paola et al., 1990). The fluorescent alkylating probe *N*-(1-pyrenyl)maleimide, which inhibits ion channel opening, labels Cys-222 of the M1 segment (Marquez et al., 1989), providing further evidence that probes affecting cation translocation properties of the receptor label the M1 domain. Thus, although it appears that the M2 segments line the channel, there is also evidence that M1 contributes to or is exposed to the channel.

In this paper, we report the results of a study to investigate binding sites on a 56-residue synthetic peptide (Tα1-56mer) containing residues 172–227 of the α-subunit of the *Torpedo* AChR. This peptide was synthesized because it contains (1) a region (residues 173–204) previously shown to bind α-Btx (Wilson et al., 1985; Wilson & Lentz, 1988) and (2) a region including the first 18 residues of the first transmembrane domain, M1. The binding of an antagonist, α-Btx, and an NCI, PCP [1-(1-phenylcyclohexyl)piperidine], to the Tα1-56mer were characterized. The results presented here demonstrate that this 56-residue peptide contains binding sites for both α-Btx and PCP, thereby supporting the suggestion these two sites are tightly coupled and part of one functional domain.

Table I: Amino Acid Sequences of *Torpedo* α1-Subunit Peptides[a]

	175 180 185 190 195 200 205 210 215 220 225
Tα1 56mer	ESGEWVMKDVRGWKHWVYYTCCPDTPYLDITYHFIMQRIPLYFVVNVIIPCLLFSF
Tα1 32mer	SGEWVMKDVRGWKHWVYYTCCPDTPYLDITYH
Tα1 23mer	FIMQRIPLYFVVNVIIPCLLFSF

α-Btx Binding Site
Low affinity PCP
Binding Site

High affinity PCP
Binding Site

[a] Peptides are designated by the number of residues. Proposed binding sites for the antagonist α-Btx and the noncompetitive inhibitor PCP are indicated.

Materials and Methods

CPZ, dibucaine, tetracaine, *d*-tubocurarine, insulin, and reagents were purchased from Sigma Chemical Co., St. Louis, MO. PCP was a gift from Dr. Robert Roth, Department of Pharmacology, Yale University School of Medicine. [^{3}H]PCP and [^{3}H]strychnine were purchased from NEN/Dupont Co., Boston, MA, and [^{3}H]muscimol was from Amersham Corp., Arlington Heights, IL. α-Btx was obtained from Miami Serpentarium (Salt Lake City, UT) and iodinated with ^{125}I by the chloramine T method (Wang & Schmidt, 1980). The monoiodinated α-Btx used in these experiments was separated from diiodinated α-Btx by using a CM-25 ion-exchange column. The initial specific activity of the labeled toxin was always greater than 500 cpm/fmol. RNA-binding protein was a gift of Dr. Kenneth Williams, Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine.

Synthetic Peptides. All AChR and other peptides used in these studies were synthesized by the Protein Chemistry Facility, Department of Molecular Biology and Biophysics, Yale University. The peptides correspond to portions of the *Torpedo* α1-subunit and are designated by the total number of residues (Table I). All peptides were synthesized with their amino- and carboxy-termini blocked. The integrity of the peptide sequences was determined by amino acid composition analysis and by reverse-phase high-performance liquid chromatography. Molecular weight of the peptides was confirmed by mass spectroscopy. Peptides were purified by reverse-phase high-pressure liquid chromatography with a Vydac C_4 column and a gradient of 0–80% acetonitrile/0.05% trifluoroacetic acid. The eluted peptide was lyophilized and resuspended in 50% acetonitrile/H_2O and 0.005% trifluoroacetic acid to a final concentration of 5 mg/mL.

Binding of ^{125}I-α-Btx to Synthetic Peptides. ^{125}I-α-Btx binding to synthetic peptides was measured by a solid-phase assay. The peptides dissolved in 50% acetonitrile/H_2O and 0.005% trifluoroacetic acid were diluted into H_2O to a concentration of 50 μg/mL. Wells of a 96-well microtiter plate were then coated by evaporation overnight at 45 °C with 100 μL (5 μg) of peptide solution. Wells were then quenched with 200 μL of 2% bovine serum albumin (BSA) for 1 h at room temperature. After being quenched, the wells were aspirated and incubated with ^{125}I-α-Btx in phosphate buffer (PB)/0.2% BSA for 2 h. For some experiments, sodium dodecyl sulfate (SDS) was included in the incubation medium. Wells were washed four times with PBS, and bound radioactivity was

removed by adding 100 μL of 0.25 N NaOH/2.5% SDS and swabbing each well twice with cotton-tipped applicators and placing them in a tube. Radioactivity was counted in a γ counter. Background binding to wells lacking peptide was subtracted from the binding in the presence of peptide. Competitive binding assays with unlabeled α-Btx, *d*-tubocurarine, and NaCl were performed as described previously (Wilson et al., 1988). From these competition experiments, the binding affinities were determined by measuring the concentration of unlabeled ligand that resulted in a 50% reduction in the binding of ^{125}I-α-Btx (IC_{50} value). IC_{50} values were determined from logit-log plots of the competition data (Rodbard & Frazier, 1975).

Equilibrium saturation experiments were performed in the absence or presence of 0.02% SDS in PB/BSA in the solid-phase assay system described above. Cold toxin was added to ^{125}I-α-Btx to reduce the specific activity. Incubation was performed for 8 h at room temperature. Equilibrium binding data were analyzed in Scatchard plots. Binding curves were fitted by linear least-squares analysis. Nonlinear curves were fitted to two linear binding curves by applying the limiting-slope technique of Hunston (1975) to the data.

Binding of [^{3}H]PCP to Synthetic Peptides. AChR synthetic peptides (2.5–10 μg) or control peptides in 300 μL of 10 mM PB, pH 7.5, and SDS (0.01–0.02%) were incubated with 29 nM [^{3}H]PCP (~100000 cpm) for 30 min at room temperature followed by a 10-min irradiation with a Mineralight short-wave ultraviolet (UV) lamp (254 nM) placed at a distance of 15 cm from the samples (Oswald & Changeux, 1981). Each sample was then blotted onto a piece of nitrocellulose (45-μM pore size) in a slot-blot apparatus and washed with 500 μL of PBS by vacuum aspiration. The filters were stained with 0.5% Ponceau S in 5% trichloroacetic acid and the portions of filters containing blotted peptide were cut out, placed in the fluorophore Cytoscint (ICN), and counted in a scintillation counter for 1 min. Binding to peptides could also be detected without UV irradiation, but because the signal was higher after photoincorporation, the latter procedure was used in most experiments. Initial experiments were performed to determine the optimum conditions for detecting the binding of [^{3}H]PCP to the receptor peptides. The effect of SDS on binding of [^{3}H]PCP to the peptides was tested and the concentration of SDS producing maximum enhancement was determined. Equilibrium binding experiments were performed in the presence of 0.02% SDS in PB/BSA. The specific activity of [^{3}H]PCP was reduced by addition of unlabeled PCP. The peptides were incubated in solution with increasing concentrations of [^{3}H]PCP for 2 h at room temperature followed by UV irradiation for 10 min. After incubation, an aliquot of each sample was removed and counted in a scintillation counter to determine the amount of free [^{3}H]PCP. The samples were blotted onto nitrocellulose and the filter was washed as described above to determine the amount of bound radioactivity. Equilibrium binding data were analyzed by Scatchard plots. The limiting-slope technique was applied to the nonlinear curves. In competition experiments, various concentrations of other NCIs were added to the [^{3}H]PCP solution prior to incubation with the Tα1-56mer peptide. The incubation time prior to irradiation for these competition experiments was 10 min. The effect of CPZ (200 μm) and α-Btx (10^{-5} M) on [^{3}H]PCP binding to all of the peptides was tested after preincubation and incubation with the inhibitors.

Results

α-Btx Binding to Tα1-56mer. ^{125}I-α-Btx binding to the Tα1-56mer was demonstrated by using a solid-phase radioassay in which the peptide was adsorbed to the wells of polystyrene microtiter plates and incubated with labeled toxin. As observed previously for the Tα1-32mer (Wilson & Lentz, 1988), binding of α-Btx to the Tα1-56mer was enhanced by SDS. Enhancement of binding was strongly dependent on the concentration of SDS and was maximal between 0.015% and 0.02% SDS. No α-Btx binding to the Tα1-23mer was detected with the solid-phase assay.

Table II: [^{3}H]PCP Photoaffinity Labeling of AChR Synthetic Peptides[a]

	cpm bound		% inhibition	
	− SDS	+ SDS	CPZ	α-Btx
Tα1-56mer	1025	7585	90	39
Tα1-23mer	550	9845	81	17
Tα1-32mer	211	2632	91	100

[a] Peptides (10 μg) were incubated for 30 min at room temperature with 29 nM [^{3}H]PCP ± SDS (0.01%) followed by 10 min of UV light irradiation at 254 nM. The samples were then blotted onto nitrocellulose and the filters washed and counted. Background cpm in the absence of peptide was subtracted from experimental values. Each determination was performed in duplicate. Peptides were preincubated for 1 h at room temperature with excess CPZ (200 μM) or α-Btx (10^{-5} M) prior to addition of [^{3}H]PCP. The percent reduction in [^{3}H]PCP binding in the presence of these agents was calculated.

Equilibrium saturation binding studies were performed for the binding of ^{125}I-α-Btx to the Tα1-56mer peptide by using the solid-phase assay. Saturable binding of ^{125}I-α-Btx to Tα1-56mer adsorbed onto plastic wells was obtained. Scatchard analysis of α-Btx binding to the peptide in the absence of SDS yielded a nonlinear plot indicating the presence of two binding components: a minor component with an apparent dissociation constant (K_D) of 1.8 nM, consisting of 6.3% of the total binding sites, and a major component with an apparent K_D of 23 nM, involving 93.7% of the sites (data not shown). In the presence of 0.02% SDS, a linear plot was obtained and Scatchard analysis of the equilibrium binding data revealed one class of binding sites with an apparent K_D of 3.5 nM. The ability of cholinergic ligands to inhibit binding of ^{125}I-α-Btx to the Tα1-56mer was tested. Unlabeled α-Btx, *d*-tubocurarine, and NaCl inhibited binding of labeled toxin with IC_{50} values of 5.0×10^{-8} M, 5.9×10^{-5} M, and 7.9×10^{-2} M, respectively (data not shown).

PCP Binding to the AChR Peptides. Studies were performed to determine whether PCP bound to the α-subunit peptides. Binding was first tested by using the solid-phase radioassay; however, no appreciable binding was detected due to very high background levels. After comparing several assays, a filter assay employing a slot-blot apparatus and nitrocellulose was found to yield a high signal/background ratio (5–10:1). This assay also retained 85% of the labeled peptide on the basis of amino acid analysis of the sample transferred to PVDF membranes. In the absence of SDS, [^{3}H]PCP exhibited the highest amount of binding to the Tα1-56mer, less to the Tα1-23mer, and even less binding to the Tα1-32mer (Table II). The effect of SDS on the binding of [^{3}H]PCP to the peptides was tested. SDS markedly enhanced binding to all of the α-subunit peptides (Table II). In the presence of 0.01% SDS, PCP binding to the Tα1-56mer and Tα1-23mer was comparable while that to the Tα1-32mer was considerably less. The enhancement of binding was strongly dependent on the concentration of SDS, with the maximum effect occurring at 0.02% SDS (Figure 1).

A time course of association was performed by incubating [^{3}H]PCP with the Tα1-56mer for increasing periods of time prior to a 10-min UV irradiation. Equilibrium binding was reached with a 10-min preincubation. Equilibrium saturation

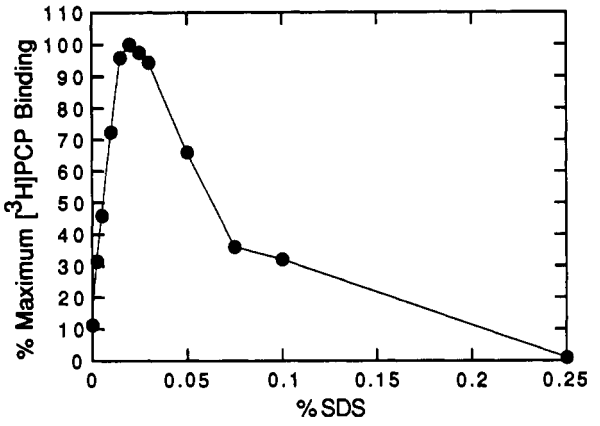

FIGURE 1: Effect of SDS on the binding of [^{3}H]PCP to Tα1-56mer. Peptide (5 μg) was incubated with various concentrations of SDS and [^{3}H]PCP for 30 min at room temperature. The samples were irradiated with UV light for 10 min in the dark. The samples were then blotted onto nitrocellulose and the filters washed and counted in a scintillation counter as described under Materials and Methods. Net cpm was calculated by subtracting cpm of [^{3}H]PCP bound in absence of peptides at the same SDS concentrations from binding in the presence of peptide. Values are the mean of triplicate determinations.

binding studies for [^{3}H]PCP binding to the Tα1-56mer were performed in the presence of 0.02% SDS. A nonlinear plot revealing the existence of at least two saturable sites was observed (Figure 2A). Scatchard analysis of the saturation data according to a two-site model yielded a low-affinity site with an apparent K_D of 4.6 μM and a high-affinity site with a K_D of 0.4 μM. These values were determined by applying the limiting-slope technique (Hunston, 1975) to the Scatchard data. In contrast to the Tα1-56mer, Scatchard analysis of saturation binding data for the Tα1-23mer resulted in a linear plot (Figure 2B). The apparent K_D for PCP binding to the Tα1-23mer was 0.27 μM, comparable to the high-affinity binding observed with the Tα1-56mer.

It should be noted that true dissociation is not being determined by these procedures because irreversible binding is being measured. Thus, the equilibrium binding studies are better termed concentration dependence studies. These procedures therefore allow comparison of the relative affinities of PCP for the peptides.

Several experiments were performed to determine the specificity of [^{3}H]PCP binding to the α-subunit peptides. The effect of other NCIs and cholinergic ligands on binding was tested. First, binding of PCP to the Tα1-56mer was inhibited by CPZ, tetracaine, and dibucaine with IC_{50} values of 6.3×10^{-5} M, 4.2×10^{-6} M, and 2.7×10^{-4} M, respectively (Figure 3). These agents also inhibited the binding that is observed without UV irradiation. The ability of cold α-Btx to block the binding of PCP to the Tα1-56mer, Tα1-32mer, and Tα1-23mer was also determined. Cold toxin decreased the binding of PCP to the Tα1-56mer, slightly decreased binding to the Tα1-23mer, and completely abolished binding to the Tα1-32mer (Table II). In contrast, excess CPZ effectively competed binding to all three peptides (Table II). Finally the effects of carbamylcholine, *d*-tubocurarine, and decamethonium on PCP binding to the Tα1-56mer were tested. These agents partially inhibited PCP binding at high concentrations ($>10^{-4}$ M) and no significant enhancement of binding was observed at low concentrations (data not shown).

Second, [^{3}H]PCP was incubated in the presence of 0.02% SDS and irradiated with the following peptides: bovine pancreas insulin, residues 175–203 of rabies virus glycoprotein peptide, a 56-residue peptide corresponding to the primary structure of the p10 retroviral RNA-binding protein of murine leukemia virus, residues 188-201 of the $GABA_A$ receptor β subunit, and residues 189–209 of the glycine receptor 48K subunit. In all cases the signal observed with these peptides did not exceed the background signal in the absence of peptide.

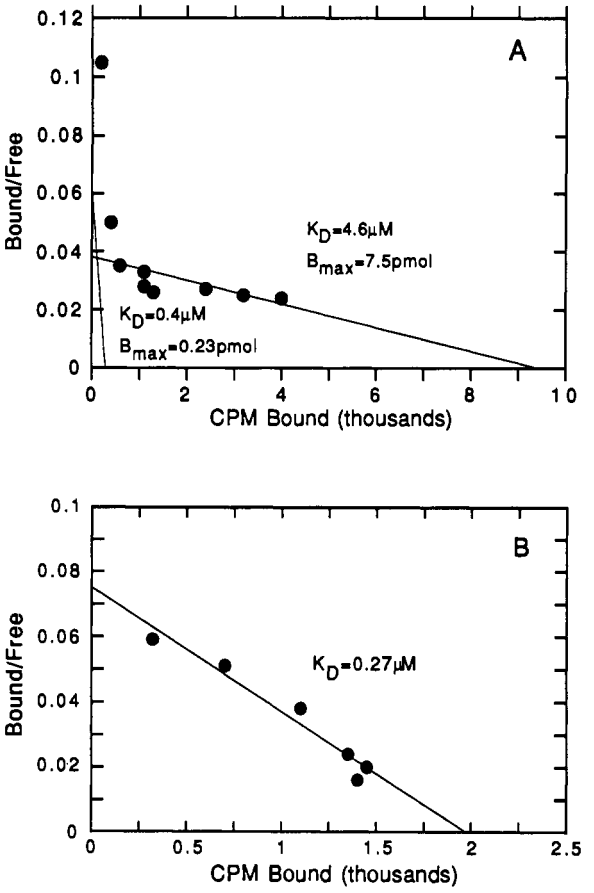

FIGURE 2: Scatchard analysis of the binding of [^{3}H]PCP to the Tα1-23mer. Peptide (5 μg) in the presence of 0.02% SDS was incubated with various amounts of [^{3}H]PCP for 2 h at room temperature followed by 10 min of UV light irradiation in the dark. An aliquot was removed to determine the free [^{3}H]PCP concentration. The samples were blotted onto nitrocellulose and the filters washed and counted. Background binding in the absence of peptide was subtracted from total binding in the presence of peptide. Points are the average of two experiments of three replicates each. (A) [^{3}H]PCP binding to Tα1-56mer. Specific activity of [^{3}H]PCP equals 1.13 Ci/mmol. (B) [^{3}H]PCP binding to Tα1-23mer. Specific activity of [^{3}H]PCP equals 1.23 Ci/mmol.

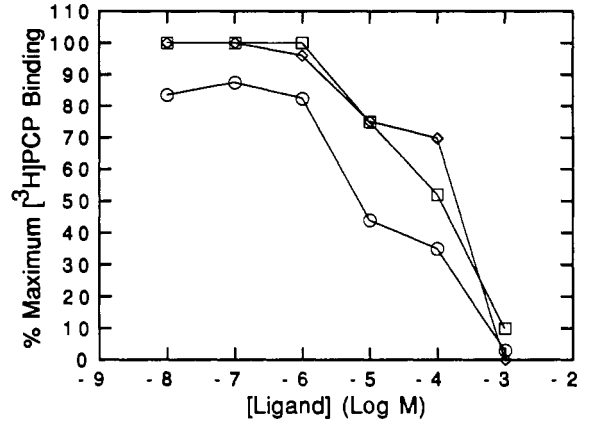

FIGURE 3: Competition of [^{3}H]PCP binding to Tα1-56mer. Tα1-56mer (5 μg) in the presence of 0.02% SDS was incubated with [^{3}H]PCP and a range of concentrations of unlabeled NCIs: tetracaine (circles), CPZ (squares), or dibucaine (triangles). Binding was determined (three separate experiments, each in duplicate) by using a slot blot assay as described under Materials and Methods.

Third, it was determined whether ligands of other receptors could be photoincorporated into the AChR Tα1-56mer. The Tα1-56mer was incubated and irradiated in the presence of [^{3}H]muscimol, a GABA receptor agonist, and [^{3}H]strychnine, a glycine receptor antagonist, under the same conditions and concentrations as used for [^{3}H]PCP binding. No incorporation of these agents into the Tα1-56mer was observed.

Discussion

Synthetic peptides have proven useful in localizing α-Btx binding sites on the AChR [see Lentz and Wilson (1988)]. Previous studies by this laboratory have described binding of α-Btx to synthetic peptides situated between residues 173 and 204 (Wilson et al., 1988). The present results show that a synthetic peptide comprising residues 172–227 of the α-subunit of the *Torpedo* AChR contains three binding sites, one for α-Btx and two for the NCI PCP. With respect to α-Btx, the Tα1-56mer behaved in a manner nearly identical with that of the Tα1-32mer (residues 173–204) (Wilson & Lentz, 1988). In the absence of SDS, two binding components with apparent K_Ds of 1.8 nM and 23 nM were observed for the 56mer, compared to apparent K_Ds of 4.2 nM and 63 nM for the 32mer. In the presence of SDS, only the high-affinity component was detected; K_D = 3.5 nM for the 56mer and K_D = 7.8 nM for the 32mer. In the case of α-Btx binding to the Tα1-32mer, it was concluded that SDS stabilizes a conformation of the peptide that is conducive to high-affinity binding (Wilson & Lentz, 1988). These affinities compare with a K_D of 4.1×10^{-10} M for α-Btx binding to the intact native receptor (Wilson & Lentz, 1988) and an IC_{50} value of 12 nM for competition of ^{125}I-labeled AChR binding to α-Btx by isolated α-subunit (Wilson et al., 1988). Thus, the affinity of α-Btx for denatured α-subunit is comparable to the low-affinity binding, which represents the major binding component in the absence of SDS, to the 32mer and 56mer. α-Btx binding could not be detected to the Tα1-23mer, residues 205–227. Because of the similarity in the binding characteristics of the Tα1-56mer and Tα1-32mer and the absence of binding to the Tα1-23mer, it is concluded that the α-Btx binding determinants on the 56mer are located between residues 172 and 204. The additional residues beyond 204 do not significantly enhance binding. Because of the similarity between the affinity of α-Btx for the Tα1-56mer and the apparent affinity for isolated, denatured α-subunit, it is concluded that this region represents the major toxin-binding component on the α-subunit. Another minor binding component located between residues 55 and 74 has been reported to contribute to the α-Btx binding site (Conti-Tronconi et al., 1990).

These studies demonstrate binding of the NCI PCP to the Tα1-56mer. In addition, they show that there are two sites, one of low affinity and one of high affinity, on distinct regions of the peptide (apparent K_D = 4.6 μM and 0.4 μM, respectively). As with α-Btx, SDS enhanced the total binding of PCP. PCP binding to the control peptides was negligible in the presence of SDS, indicating SDS does not nonspecifically increase binding. In contrast, two binding components for PCP binding to the Tα1-56mer were detected in the presence of SDS. Comparison of the data for PCP binding to the Tα1-56mer, Tα1-32mer, and Tα1-23mer strongly indicates that the two binding components are located in different regions. [^{3}H]PCP binding could be detected to all three peptides. Binding was greater to the Tα1-23mer than to the Tα1-32mer. Binding of PCP to the Tα1-23mer was comparable to that to the Tα1-56mer in the presence of SDS, indicating the major binding component is located on the 23mer. CPZ at concentrations sufficient to block high- and low-affinity binding abolished PCP binding to all three peptides. α-Btx, in contrast, had little or no effect on binding to the Tα1-23mer, partially inhibited binding to the Tα1-56mer, and completely abolished binding to the Tα1-32mer. This observation suggests that the minor PCP-binding component is located in the α-Btx binding site between residues 173 and 204. Finally, equilibrium binding of PCP to the Tα1-23mer revealed a single site whose affinity was nearly identical with that of the high-affinity site of the Tα1-56mer. It is concluded, therefore, that a major NCI site of high affinity is located between residues 205 and 227 and a minor NCI site of low affinity is located between residues 173 and 204.

Several observations indicate the binding of [^{3}H]PCP to the AChR peptides is specific. First, photoincorporation of PCP into the Tα1-56mer could be competed by CPZ, tetracaine, and dibucaine, other NCIs. These agents also inhibited the binding observed in the absence of UV irradiation, indicating the effect of these agents is not due to the absorption of UV light. Second, [^{3}H]PCP could not be photoincorporated into five other peptides in which all of the residues present in the Tα1-56mer are represented. Two of these peptides are derived from proteins of the same family of ion channel receptors as the AChR. The $GABA_A$ receptor peptide is homologous to residues 182–195 of the AChR α-subunit and the glycine receptor peptide is homologous to residues 179–200. Third, [^{3}H]muscimol and [^{3}H]strychnine, which can be photoincorporated into their respective receptors (Graham et al., 1981; Casalotti et al., 1986), did not label the AChR peptides.

The characteristics of PCP binding to the synthetic peptides were similar in several respects to binding of PCP to intact receptor. Several studies have reported two affinities for PCP binding with dissociation constants of 0.10 μM and 3.1 μM (Eldelfrawi et al., 1980), 0.7 μM and 3.5 μM (Oswald & Changeux, 1981), 5.1 μM and 170 μM (Heidmann et al., 1983), and 6–9 μM and 85 μM (Haring & Kloog, 1984). These compare with the values of 0.4 μM and 4.6 μM observed for binding to the Tα1-56mer. Binding to the low-affinity site on the receptor was inhibited by cholinergic agents and was considered to be to the ACh binding site (Haring & Kloog, 1984). Similarly, in the case of the peptides, low-affinity binding of PCP occurring between residues 173 and 204 was inhibited by α-Btx. The high-affinity binding between residues 205 and 227 was not affected by α-Btx. Unlike the intact receptor, PCP binding to the peptide was not significantly increased by cholinergic agonists. This is not surprising since the effect of agonists on the receptor is to open the channel, exposing the high-affinity sites for some NCIs (Oswald et al., 1983). Since the site is exposed on the peptide, it would not be expected that agonists would increase binding. In addition, PCP binds to the receptor in the closed or open channel state, indicating there is a preexisting site on the closed receptor (Albuquerque et al., 1980; Eldefrawi et al., 1980; Karpen & Hess, 1986).

The present findings suggest that a site of high-affinity PCP binding is present on the α-subunit of the AChR. Other studies have reported binding of NCIs to the α-subunit. Covalent labeling of the AChR with PCP has been reported to label δ- and α-subunits (Oswald & Chaugeux, 1981). A derivative of PCP, azidophencyclidine, was found to label the α- and β-subunits (Haring et al., 1984). Labeling of the α-subunit was inhibited by tetracaine and PCP but not by α-Btx. It was concluded that the high-affinity PCP site is located between the α- and β-subunits and that PCP attaches to both (Haring et al., 1984). Azidophencyclidine labeled an 18-kDa V8 protease fragment of the α-subunit that also bound

α-Btx (Mosckovitz et al., 1987). Labeling of the fragment occurred in both the presence and absence of carbamylcholine. Chlorpromazine binds to all four subunits (Oswald & Changeux, 1981). Quinacrine azide labels the α and β chains (Cox et al., 1985). Meproadifen mustard labeled a 20-kDa fragment of the α-subunit beginning at Ser-173 (Pedersen et al., 1986). Thus, there is evidence that some NCIs bind to the α-subunit in the intact receptor, although labeling of other subunits has been demonstrated. The fact that all four subunits can be labeled indicates all subunits contribute to the NCI binding site.

There is considerable evidence that NCIs label transmembrane segment M2 of receptor subunits. CPZ (Giraudat et al., 1986) and triphenylmethylphosphonium (Hucho et al., 1986; Oberthür et al., 1986) have been shown to label Ser-262 of *Torpedo marmorata* δ-subunit. Triphenylmethylphosphonium also labeled Ser-254 in the β-subunit and Ser-248 in the α-subunit (Hucho et al., 1986). In addition, CPZ labeled Ser-254 and Leu-257 of the β-subunit (Giraudat et al., 1987) and Ser-248 of the α-subunit (Giraudat et al., 1989). The residues labeled by the NCIs are located in the M2 transmembrane segment, therefore suggesting that M2 faces and lies within the ion channel. Site-directed mutagenesis and expression of the mutant *Torpedo* AChR in *Xenopus* oocytes followed by single-channel recording analysis was another method used to identify amino acids important for a functional channel. These studies provided evidence that alteration of charged residues on either side of M2 of all subunits affected cation conductance (Imoto et al., 1988). Mutation of serine residues in M2 of α-, β-, and δ-subunits decreased the affinity of a cationic open-channel blocker, QX-222, and decreased outward single-channel currents (Leonard et al., 1988). Finally, two 23-residue synthetic peptides comprising M1 and M2 of the *Torpedo* δ-subunit were compared in their ability to form ionic channels when reconstituted in phosphatidylcholine bilayers. The M1 peptide did not form channels while the M2 peptide formed channels with properties similar to those of the AChR channel (Oiki et al., 1988). Therefore, NCI photoaffinity labeling experiments and functional analysis of mutant AChR provide evidence for the placement of M2 in the ion channel.

As described above, several studies have shown that NCIs label a serine residue in M2. A serine residue is present at position 226 of M1 and could represent a site of labeling of the synthetic peptides by PCP. It is possible that PCP could be nonspecifically cross-linked to serine residues in the synthetic peptides. However, PCP binding to the Tα1-32mer, which contains a serine residue, was considerably less than to the Tα1-23mer and did not occur at all to the control peptides containing serine residues. These results indicate that if PCP is cross-linked to a serine residue, serine alone is insufficient for binding and surrounding residues are necessary for recognition and binding.

Other evidence indicates that residues within transmembrane segment M1 are labeled by NCIs and may be exposed to the channel pore. Di Paola et al. (1990) reported that [^{3}H]quinacrine azide labeling occurred on cyanogen bromide fragment, residues 208–243 of the α-subunit, which contains transmembrane segment M1. A possible explanation of the difference from the studies described above in which M2 was labeled is that only in the case of quinacrine azide was labeling performed in the open state of the AChR (Di Paola et al., 1990). Our results are in agreement with those of Di Paola et al. (1990) and show that a high-affinity NCI binding site is present between residues 205 and 227 of the α-subunit. These observations support the suggestion that a single exon encoding residues 161–239 (Noda et al., 1983) forms a structural and functional domain mediating rapid coupling between the ACh binding site and the channel gate (Di Paola et al., 1990).

The presence of both ACh and NCI binding sites on the Tα1-56mer is consistent with estimates of the distances between these sites in the intact receptor. Fluorescence energy transfer measurements between energy donors bound to the agonist sites and an energy acceptor bound to the NCI site indicate that the distance of the NCI site from the agonist sites is 21–35 Å for one site and 22–40 Å for the other (Herz et al., 1989). Cys-192 and possibly also Cys-193 are labeled by MBTA and lie close to the ACh binding site (Kao et al., 1984). The studies of Di Paola et al. (1990) show that a high-affinity NCI site is located between residues 208 and 243 of the α-subunit and our studies indicate a site is located within residues 205–227. Our studies also indicate that this site for high-affinity binding is in close proximity to the site for agonist/antagonist binding (residues 172–204).

Dani (1989) proposed a receptor model in which the M1 helices lie just behind the M2 helices and are exposed to the open pore at the interstices of the M2 helices near the outer vestibule. It was also noted that M1 is highly conserved among all members of the ligand-gated receptor channel family, i.e., AChR, GABA receptor, and glycine receptor, and contains a proline residue in the middle (position 221). The proline residue, which would produce a kink in the α-helix structure of M1, and neighboring hydrophobic residues confer flexibility on this region. The high-affinity PCP binding site is considered to be located in the channel of the receptor (Albuquerque et al., 1980; Eldefrawi et al., 1980; Oswald et al., 1983; Haring et al., 1984). Thus, the presence of a NCI binding site within residues 205–227 of the α-subunit indicates this region is in close proximity to the channel. The location of this site adjacent to the agonist binding site and the potential for flexibility present within M1 suggest that this region of the α-subunit could be involved in gating of the channel. Local conformational changes occurring as a result of ACh binding (Karlin, 1969; Kao & Karlin, 1986) may be propagated sequentially along the polypeptide chain from the ACh binding site to M1. The latter may affect adjacent transmembrane segments (M2), resulting in opening of the channel. It has been shown that the overall conformational changes in the AChR upon agonist binding are relatively small (McCarthy & Stroud, 1989). The localization of the agonist binding site and gating mechanism to a relatively small functional unit of the receptor is consistent with the absence of global conformational changes in the receptor in response to agonist binding.

Registry No. α-Btx, 11032-79-4; PCP, 77-10-1; CPZ, 50-53-3; tetracaine, 94-24-6; dibucaine, 85-79-0.

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Partitioning of Fluorescent Phospholipid Probes between Different Bilayer Environments. Estimation of the Free Energy of Interlipid Hydrogen Bonding†

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ABSTRACT: Fluorescence spectroscopy has been used to monitor the partitioning of a series of exchangeable neutral phospholipid probes, labeled with carbazole, indolyl or diphenylhexatrienyl moieties, between large unilamellar vesicles containing 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) or *N*-hexadecyl-*N*-(9-octadecenyl)-*N*,*N*-dimethylammonium chloride (HODMA). Phosphatidylethanolamine (PE) probes desorb from POPC-containing vesicles at markedly slower rates than do phosphatidylcholine (PC) probes with the same acyl chains. The rate of probe desorption from such vesicles is progressively enhanced by successive *N*-methylations of the amino group but not by methylation of C-2 of the ethanolamine moiety, a modification that leaves unaltered the hydrogen-bonding capacity of the polar headgroup. By contrast, the rates of desorption of different probes (with the same acyl chains) from HODMA or from DOTAP vesicles are much more comparable and reflect no clear systematic influence of the headgroup hydrogen-bonding capacity. Equilibrium-partitioning measurements indicate that the relative affinities of different probes for PC-rich vesicles, in competition with HODMA or DOTAP vesicles, increase with increasing hydrogen-bonding capacity of the probe headgroup in the order PC < *N*,*N*-dimethyl PE < *N*-methyl PE < PE ≈ phosphatidyl-2-amino-1-propanol. From such partitioning data, we estimate that interlipid hydrogen-bonding interactions (in competition with lipid–water interactions) contribute roughly −300 cal mol^{-1} to the free energy of a PE molecule in a hydrated liquid-crystalline phospholipid bilayer; this free-energy contribution is somewhat smaller, but still significant, for *N*-mono- and dimethylated PE's.

A number of biologically important membrane lipids, including the aminophospholipids, glycolipids, and sphingolipids, possess the ability to act as hydrogen-bond donors. Interactions between such molecules and like or unlike species that act as hydrogen-bond acceptors may play an important role in determining membrane stability, surface properties, and lipid lateral organization (Boggs, 1980, 1987; Curatolo, 1986; Thompson & Tillack, 1985; Seddon, 1990).

While it is virtually certain that membrane lipids with hydrogen-bond-donating abilities participate in intermolecular hydrogen bonds of some type in lipid bilayers, the relative strengths of lipid–lipid and lipid–solvent hydrogen-bonding interactions in such systems are by no means clear. The distinctive thermotropic behavior of lipid species such as phosphatidylethanolamine (PE)[1] (Chang & Epand, 1983; Mantsch et al., 1983; Seddon et al., 1983; Wilkinson & Nagle, 1984; Seddon, 1990) and various glycolipids (Bunow, 1979; Freire et al., 1980; Sen et al., 1981; Ruocco et al., 1981; Curatolo, 1982, 1986; Koshy & Boggs, 1983; Maggio et al., 1985; Hinz et al., 1985; Curatolo & Jungawala, 1985; Mannock et al., 1988, 1990) has been attributed at least in part to the hydrogen-bond-donating abilities of their polar headgroups, and X-ray diffraction studies have provided evidence for hydrogen bonding between the headgroups of adjacent lipid molecules in anhydrous crystals of PE (Hitchcock et al., 1974) and of cerebroside (Pascher & Sundell, 1977). However, no direct estimate of the strength of such interlipid hydrogen bonds has been obtained to date. The strength of such interactions is particularly difficult to estimate a priori for the case of hydrated lipid bilayers, where any potential interlipid hydrogen bonds must compete with hydrogen-bonding interactions between the lipid polar headgroups and water.

We have previously used exchangeable phospholipid analogues, labeled with fluorescent reporter groups, to evaluate

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[1] Abbreviations: 8/(11-Carbazole)-PC (-PE), 1-octanoyl-2-[11′-(carbazol-9″-yl)undecanoyl]-*sn*-glycero-3-phosphocholine (-*sn*-glycero-3-phosphoethanolamine); (12-DABS)-18 PC, 1-palmitoyl-2-[12-[[[4-[[4-(dimethylamino)phenyl]azo]phenyl]sulfonyl]methylamino]octadecanoyl]-*sn*-glycero-3-phosphocholine; DOTAP, 1,2-dioleoyloxy-3-(trimethylammonio)propane; 10/(3-DPH)-PC (-PE), 1-decanoyl-2-[3-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]propanoyl]-*sn*-glycero-3-phosphocholine (-*sn*-glycero-3-phosphoethanolamine); HODMA, *N*-hexadecyl-*N*-(*cis*-9-octadecenyl)-*N*,*N*-dimethylammonium chloride; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; POPC, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphocholine; POPG, 1-hexadecanoyl-2-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phosphoglycerol; (16-TNP)-16 PC, 1-hexadecanoyl-2-[16-(trinitrophenylamino)hexadecanoyl]-*sn*-glycero-3-phosphocholine.

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