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Specific [^{3}H]phencyclidine binding in rat central nervous system

(ketamine/drug receptors/hallucinogens/drug abuse)

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ABSTRACT [^{3}H]Phencyclidine (PCP) bound specifically and with high affinity (K_d = 0.15 μM at pH 7.4) to a single saturable class of binding sites in rat brain membrane preparations. Specific binding constituted approximately 70% of total binding at 0°C and 33% of total binding at 37°C (at 10 nM [^{3}H]PCP). Bound [^{3}H]PCP could be displaced by nonradioactive PCP, a series of its derivatives, and the psychotomimetic opiate *N*-allylnorcyclazocine (SKF 10,047) with relative potencies that closely paralleled those determined in animal behavioral tests. Muscarinic cholinergic ligands inhibited [^{3}H]PCP binding, but only at 0.1 mM and in rank order at variance with that for binding to muscarinic sites or for pharmacological potencies. Other drugs, including opiates other than SKF 10,047, were unable to displace specifically bound [^{3}H]PCP at 0.1 mM. [^{3}H]PCP binding was most enriched in crude synaptosomal subcellular fractions, and was about three times higher in hippocampus (region of highest density) than in cervical spinal cord (region of lowest density). Trypsin and Pronase reduced specific [^{3}H]PCP binding. Thus, PCP may exert its effects on the central nervous system via binding to specific brain receptor sites.

Phencyclidine [*N*-(1-phenylcyclohexyl)piperidine; PCP], introduced in the 1950s as a general anesthetic, was rejected for use in humans, primarily because of its psychotomimetic effects (1–3). Although it is still considered a useful veterinary anesthetic, its widest present interest is as a drug of abuse (4–6). The ease of synthesis and administration of PCP have contributed to its popularity among illicit users (5, 7).

Subanesthetic doses of PCP in humans have been found to cause effects primarily upon the cardiovascular system (hypertension, tachycardia) and the central nervous system. The latter include nystagmus, gait ataxia, muscle rigidity, characteristic electroencephalographic changes, stereotypy, and psychotomimetic effects (5, 7). PCP-induced psychotic states, usually associated with chronic use, are of particular interest because of a close resemblance to schizophrenia; symptoms include agitation, auditory hallucinations, thought disorder, and paranoid delusions, typically in the absence of a prior psychiatric history (8, 9). Thus, an understanding of PCP's mode of action might provide insight into mechanisms underlying psychotic states.

Previous neuropharmacological findings have included inhibition by PCP and its derivatives of uptake of catecholamines and indoleamines by brain tissue (10–12), anticholinergic properties (13), inhibition of acetylcholinesterase (13, 14), and binding to brain muscarinic and opiate receptor sites (15). Attempts to apply these findings to the treatment of PCP-induced psychotic states have in general proven unsuccessful (4).

We have chosen to investigate the mechanism of action of PCP by studying the interaction of [^{3}H]PCP with central nervous tissue. Our data indicate that [^{3}H]PCP binds specifically and with high affinity to discrete sites on rat brain membranes. We propose that some of the central nervous actions of PCP and its derivatives may be mediated via this binding.

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MATERIALS AND METHODS

Tissue Preparation. Male Sprague–Dawley rats (150–200 g) were decapitated, and the brains (or brain regions, for regional binding studies) were rapidly removed and homogenized in 33 vol of 50 mM Tris·HCl, pH 7.4, at 4°C with a Brinkmann Polytron (setting 5, 45 sec). The homogenate was centrifuged at 30,000 × *g* for 15 min. The resulting pellet was resuspended in 100 vol of 50 mM Tris·HCl, pH 7.4, at 4°C, centrifuged again at 30,000 × *g* for 15 min, and resuspended in 100 vol of the same buffer. Membrane protein concentration was determined by the method of Lowry *et al.* (16).

Subcellular nuclear (P_1) fractions and crude mitochondrial–synaptosomal (P_2) fractions were prepared by the method of Gray and Whittaker (17). The P_2 pellet was resuspended in 33 vol of distilled water by homogenization and further fractionated into the mitochondria–myelin and crude synaptic membrane fractions according to the method of Zukin *et al.* (18). Then, suspensions of the resulting pellets were prepared from each fraction by the procedure described above.

Binding Assay. Aliquots of freshly prepared homogenate (1.0 ml, 0.5 mg of protein) in 50 mM Tris·HCl buffer, pH 7.4, were incubated in triplicate at 4°C for 45 min with 7.0 nM [^{3}H]PCP (420,000 cpm) alone or in the presence of 0.1 mM PCP or other indicated drugs. Free ligand was separated from membrane-bound [^{3}H]PCP by filtration under reduced pressure through GF/B glass fiber filters (Whatman). The filters were rapidly washed with two aliquots of 10 ml of 50 mM Tris·HCl, pH 7.4, 4°C. Filters were then transferred to Aquasol/toluene (2:1, vol/vol) and assayed by liquid scintillation spectrometry (Intertechnique ABAL SL 40) at a counting efficiency of approximately 50%.

Materials. [*piperidyl*-3,4-^{3}H]PCP (60.0 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) was obtained from New England Nuclear. Nonradioactive PCP and PCP derivatives were generously supplied by the National Institute of Drug Abuse.

RESULTS

[^{3}H]PCP Binding to Rat Brain Homogenates. Specific [^{3}H]PCP binding, defined as the total binding minus the binding in the presence of 100 μM PCP, was saturable with respect to radiolabeled ligand concentration (Fig. 1*A*). Half-maximal binding occurred at approximately 0.1 μM [^{3}H]PCP. In contrast, nonspecific binding, indicated by the binding of [^{3}H]PCP in the presence of 100 μM PCP, was not saturable and

Abbreviations: PCP, phencyclidine [*N*-(1-phenylcyclohexyl)piperidine]; QNB, quinuclidinyl benzilate; SKF 10,047, *N*-allylnorcyclazocine.

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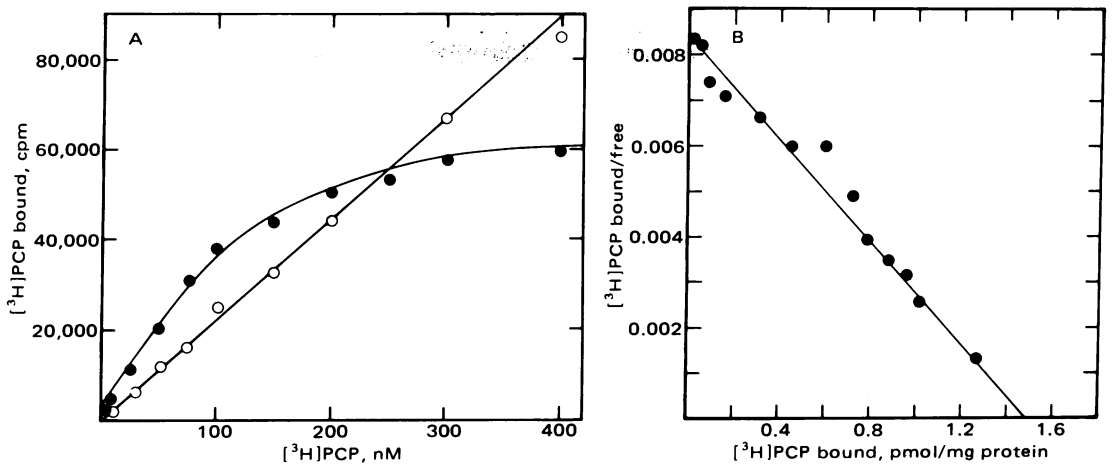

FIG. 1. Specific binding of [^{3}H]PCP to rat brain homogenates as a function of the concentration of PCP (*A*) and as a Scatchard analysis (*B*). Aliquots of homogenate (1 ml, 0.5 mg of protein) in 0.05 M Tris·HCl buffer (pH 7.4) were incubated at 4°C for 45 min with various concentrations of [^{3}H]PCP. Specific binding (●) and nonspecific binding (○) of PCP to tissue were determined as described in the text.

increased linearly with increasing [^{3}H]PCP. When these binding data were replotted in a Scatchard analysis (Fig. 1*B*), a single class of binding sites (1.48 pmol/mg of protein) was revealed with an apparent dissociation constant K_d of 0.15 μM.

Displacement of [^{3}H]PCP by nonradioactive PCP was half-maximal at 0.23 μM nonradioactive PCP (Fig. 2). This finding correlated closely with the data obtained in saturation analysis using [^{3}H]PCP alone. Nearly maximal (>90%) displacement of [^{3}H]PCP with nonradioactive PCP occurred at about 2.0 μM. The fact that half-maximal saturation occurred at similar concentrations of nonradioactive and radiolabeled PCP indicated that [^{3}H]PCP is biologically equivalent to the nonradioactive drug in terms of specific binding to nervous tissue. Specific binding of [^{3}H]PCP to rat brain homogenates was found to increase linearly between 0.05 and 0.5 mg per ml of whole rat brain protein. Binding studies were routinely performed within this linear range.

Thin-layer chromatography (1-butanol/H_2O/pyridine/acetic acid, 15:12:10:3 vol/vol) of hexane extracts of [^{3}H]PCP with homogenate incubated for 30 min as described for the binding assay gave a single sharp peak of radioactivity that cochromatographed with pure [^{3}H]PCP.

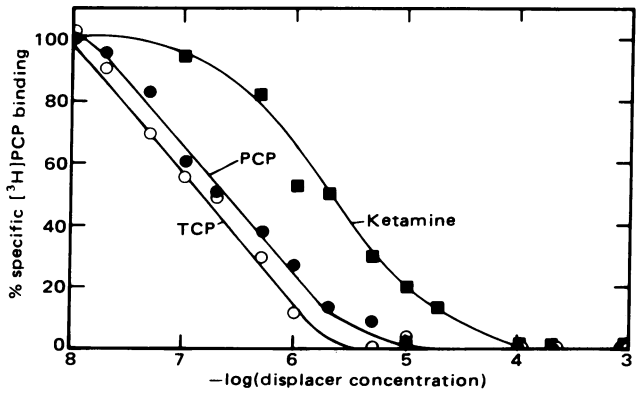

FIG. 2. Displacement of [^{3}H]PCP by nonradioactive PCP (●), *N*-[1-(2-thienyl)cyclohexyl]piperidine (TCP) (○), and ketamine (■). Rat brain homogenate suspensions (1 ml, 0.5 mg of protein per tube) were incubated with 7 nM [^{3}H]PCP (420,000 cpm) and increasing amounts of PCP, TCP, or ketamine at 4°C for 45 min. Nonspecific binding obtained in the presence of 0.1 mM PCP has been subtracted from all experimental points. Values are the means of triplicate determinations that varied less than 15%. The experiment has been replicated three times.

Effects of PCP Derivatives and Other Drugs on Specific [^{3}H]PCP Binding. See Table 1. Of the PCP analogs studied, *N*-ethyl-1-phenylcyclohexylamine (PCE), the derivative that was shown to be most active in the mouse rotarod and rat discriminative stimulus tests (19), was the most potent inhibitor of specific [^{3}H]PCP binding. The relative potencies of the PCP-like drugs tested in the binding assay correlated closely with potencies found in both the mouse rotarod test ($r = 0.81$, $P < 0.005$) (Fig. 3*A*) and the rat discriminative stimulus test ($r = 0.92$, $P < 0.001$) (Fig. 3*B*). The psychotomimetic opiate *N*-allylnorcyclazocine (SKF 10,047), the only drug structurally unrelated to PCP found to have PCP-like effects in the discriminative stimulus test (H. E. Shannon, personal communication), was also the only such drug found to inhibit [^{3}H]PCP binding.

The only other drugs that displaced specific [^{3}H]PCP binding were the muscarinic cholinergic ligands, none of which maximally displaced bound [^{3}H]PCP even at 1 mM (data not shown). Their rank order for displacement of specifically bound [^{3}H]PCP was totally different from that for their displacement of [^{3}H]quinuclidinyl benzilate (QNB) from muscarinic sites (21). Other putative neurotransmitters and central nervous system receptor agonists and antagonists (including ligands of the opiate, dopamine, glutamate, glycine, γ-aminobutyric acid, histamine, α- and β-noradrenergic, benzodiazepine, and serotonin receptors) do not compete for specific [^{3}H]PCP binding.

Kinetic Binding Data. The time course of [^{3}H]PCP association to and dissociation from rat brain membranes was examined. Specific [^{3}H]PCP binding proceeded to a fixed value and was complete in less than 2 min at 4°C. Specific binding was reversed by addition of 200-fold excess PCP. Binding declined in a monophasic, exponential manner with a half-life of approximately 1.5 min at 4°C. A dissociation rate constant ($k_{-1} = 4.8 \times 10^{-1}$ min^{-1}) was calculated (data not shown).

Further Characterization of [^{3}H]PCP Binding. In terms of subcellular distribution, binding of [^{3}H]PCP was 3-fold enriched in the P_2 fraction (153.2 fmol per mg of protein) relative to the P_1 fraction (51.0 fmol per mg of protein) and 50% enriched relative to whole brain homogenates (102.2 fmol per mg

Table 1. Relative potencies of drugs in reducing [^{3}H]PCP binding to rat brain homogenates and in behavioral effects

Drug*	Binding assay†		Relative potency (19) in behavioral tests	
	IC_{50}, μM	Relative potency	Rotarod	Discriminative stimulus
Phencyclidine (PCP)	0.23 ± 0.01	1	1	1
N-Ethyl-1-phenylcyclohexylamine (PCE)	0.14 ± 0.02	1.64	2.15	3
N-[1-(2-Thienyl)cyclohexyl]piperidine (TCP)	0.16 ± 0.01	1.44	1.29	2
1-(1-Phenylcyclohexyl)pyrrolidine (PHP)	0.20 ± 0.01	1.15	1.08	1
N-[1-(2-Thienyl)cyclohexyl]pyrrolidine (THP)	0.30 ± 0.02	0.77	1.54	1
N-(*n*-Propyl)-1-phenylcyclohexylamine (NPPCA)	0.31 ± 0.01	0.74	0.82	1
N,N-Dimethyl-1-phenylcyclohexylamine (PCDEA)	0.51 ± 0.02	0.45	1.52‡	1‡
N-Allylnorcyclazocine (SKF 10,047)	0.75 ± 0.02	0.31	—	0.29‡
Ketamine	1.00 ± 0.02	0.23	0.31	0.1
N-[1-(2-Thienyl)cyclohexyl]morpholine (TCM)	1.60 ± 0.09	0.14	0.17	0.06
1-(1-Phenylcyclohexyl)morpholine (PCM)	2.40 ± 0.04	0.10	0.18	0.1
1-Piperidinocyclohexanecarbonitrile (PCC)	5.5 ± 0.20	0.04	0.31	0
Oxotremorine	71 ± 5	0.0032		
Arecoline	80 ± 4	0.0029		
Scopolamine	92 ± 5	0.0025		
Quinuclidinyl benzilate (QNB)	900 ± 22	0.0003		
Atropine	1000 ± 50	0.0002		

* Drugs that did not displace [^{3}H]PCP binding at 100 μM: γ-aminobutyric acid, glycine, glutamic acid, serotonin, methysergide, lysergic acid diethylamide, [Met]enkephalin, naloxone, ketocyclazocine, levorphanol, β-endorphin, haloperidol, apomorphine, clonidine, alprenolol, phentolamine, histamine diphosphate, pyrilamine maleate, norepinephrine, WB 4101, diazepam, procaine, lidocaine.

† IC_{50}, concentration displacing 50% of bound [^{3}H]PCP. Results are the means from a minimum of three experiments, each carried out in triplicate, and are reported ± SEM.

‡ H. E. Shannon, personal communication.

of protein). The P_2 fraction contains mitochondrial and synaptosomal membranes, whereas the P_1 fraction contains cellular and nuclear debris (18). When the P_2 pellet was subjected to hypotonic shock and then further fractionated, the crude synaptic membrane fraction contained about 3.3 times as much PCP binding (520.4 fmol per mg of protein) as the mitochondria–myelin pellet (159.0 fmol per mg of protein) and had a specific activity 5 times that of the original homogenate.

The effects of various salts, temperatures, and protein-modifying agents were also investigated (Table 2). Sodium and potassium concentrations as high as 100 mM decreased [^{3}H]PCP binding by only about 30%. In contrast, calcium at physiological concentration (2.5 mM) lowered specific PCP binding 37%. Whereas manganese inhibited binding only 20% at 5 mM, it inhibited 34% at 20 mM. Specific binding was highest at 4°C and decreased to 70% at 22°C and to 30% at 37°C; moreover, preincubation of homogenate tissue at 37°C for 30 min decreased specific [^{3}H]PCP binding about 20% when compared with a control bound at 4°C with no prior incubation of tissue. Trypsin and Pronase decreased PCP binding about 40% at 1 mg

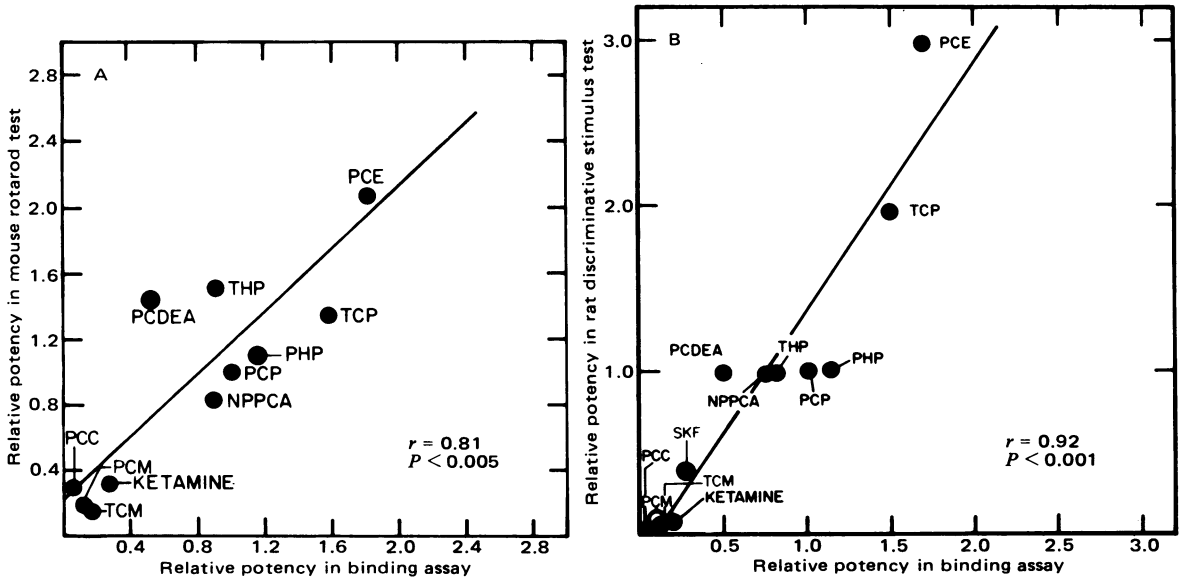

FIG. 3. Relative potencies of a series of PCP-like drugs in displacing bound [^{3}H]PCP versus the relative potencies of these compounds in the mouse rotarod test (*A*) (19) and the rat discriminative stimulus test (*B*) (ref. 19; H. E. Shannon, personal communication). See Table 1 for abbreviations. The correlations (*r*) and their significances (*P*) were determined by the Pearson product-moment correlation analysis (20).

Table 2. Effect of various reagents on specific binding of [^{3}H]PCP to rat brain homogenate

Reagent	Concentration	Specific [^{3}H]PCP binding, % of control
N-Ethylmaleimide	0.1 mM	54
	1 mM	37
Iodoacetamide	10 mM	52
Trypsin	1 mg	62
Pronase	6 μg	63
	60 μg	60
GTP	0.05 mM	90
NaCl	100 mM	64
KCl	100 mM	90
$CaCl_2$	5 mM	60
	2.5 mM	63
$MgCl_2$	10 mM	100
$MnCl_2$	5 mM	90
	10 mM	66
Heat (60°C, 15 min)		41

Reagents were added to 1 ml of brain homogenate (1.0 mg protein) during 10-min preincubation with 200 μM nonradioactive PCP. The control samples bound specifically an average of 0.1 pmol of [^{3}H]PCP. Results are the average of two experiments each carried out in triplicate.

per ml and 60 μg per ml, respectively. The sulfhydryl reagents *N*-ethylmaleimide (1 mM) and iodoacetamide (10 mM) each inhibited [^{3}H]PCP binding >40%, when compared with the control incubated under the same conditions in the absence of added agent.

Regional Distribution of Specific [^{3}H]PCP Binding. The regional distribution of [^{3}H]PCP-binding sites in rat brain was investigated (Table 3). Specific [^{3}H]PCP binding was highest in the hippocampus; binding in the cervical spinal cord was approximately one-third that in the hippocampus. Intermediate binding was observed in the hypothalamus, caudate nucleus, frontal cortex, and cerebellum, followed by the medulla/pons and amygdala. In these experiments whole homogenates of each region were utilized.

The binding of [^{3}H]PCP to homogenates of hepatic and renal tissue was also studied. These tissues exhibited some [^{3}H]PCP binding that could be displaced by nonradioactive PCP and several of its analogs. However, *N*-ethyl-1-phenylcyclohexylamine (PCE), the most potent inhibitor of brain binding, was an extremely weak inhibitor of liver or kidney binding. Conversely, ketamine, which is a weak inhibitor of brain binding, was almost equipotent to PCP in displacing [^{3}H]PCP from liver or kidney homogenates.

Table 3. [^{3}H]PCP binding in regions of rat brain

Region	Specific [^{3}H]PCP binding, fmol/mg protein
Hippocampus	150.4 ± 4.2
Hypothalamus	96.9 ± 4.1
Caudate nucleus	91.8 ± 0.3
Frontal cortex	87.1 ± 0.2
Cerebellum	79.2 ± 2.2
Medulla/pons	70.0 ± 4.7
Amygdala	66.7 ± 4.1
Cervical spinal cord	51.1 ± 3.1

Regions from fresh rat brains were dissected on ice and weighed, and homogenates were prepared as described in text. Binding assays were performed in triplicate, using membranes from each brain region and 7.0 nM [^{3}H]PCP. The mean and SEM of four separate experiments are indicated.

DISCUSSION

This study demonstrates binding of [^{3}H]PCP to a single class of sites in rat central nervous tissue. This binding is saturable and reversible, as shown by the equilibrium binding and dissociation experiments. The specificity of the binding is supported by several lines of evidence. First, binding occurs with high affinity (K_d = 0.15 μM by Scatchard analysis). Second, [^{3}H]PCP binding is inhibited by a series of PCP-like drugs. Centrally active compounds from other classes including neurotransmitters, hallucinogens, tranquilizers, narcotics, and anesthetics fail to displace [^{3}H]PCP from its binding sites even at high concentrations. Third, regional variations in binding exist, and, in subcellular fractionation experiments, binding is enriched in the crude synaptosomal fraction.

Our evidence tends to indicate the PCP-binding site to be at least in part a protein, because it is sensitive to Pronase and trypsin. The strongly inhibitory effects of pretreatment with *N*-ethylmaleimide or iodoacetamide suggest the presence of sulfhydryl groups either at or near the binding site, or at a site that critically influences the tertiary structure of the active protein. The possibility that the [^{3}H]PCP binding site is proteinaceous might also be inferred from the finding that binding decreases sharply at acidic pH values and at temperatures above 4°C, conditions that would enhance the action of endogenous proteolytic agents in the brain homogenate preparations.

[^{3}H]PCP binding to hepatic and renal homogenates was found. However, this binding failed to correlate either with binding to the central nervous system sites or with the behavioral tests, suggesting that the central nervous and peripheral binding sites differ in molecular properties and that the latter are unlikely to be of significance for the primary actions of PCP and related drugs.

The pharmacological relevance of the PCP-binding site is supported by the fact that it is selective for drugs having PCP-like behavioral effects. In addition, there is a close correlation between the potencies displayed by PCP-like drugs in the binding assay *in vitro* and those found in behavioral experiments. The ataxia estimated by the mouse rotarod test may be a good predictor of drug potency in humans because ataxia elicited even by relatively low drug doses has been noted to be a clinical sign of PCP intoxication in humans (5, 7). However, the rotarod test is potentially nonspecific, because any substance producing either ataxia or loss of motor control, by any pharmacologic or toxic mechanism, will produce positive effects. In fact, 1-piperidinocyclohexanecarbonitrile (PCC) has been found to be about 2.5 times more toxic than PCP, probably because of generation of cyanide *in vivo* after PCC administration (22). The discriminative stimulus test, on the other hand, is less likely to be influenced by nonspecific or toxic effects. In this test, rats are trained to discriminate PCP from saline in a two-choice, discrete trial avoidance task in which the task (pushing the correct lever) must be completed for a result to be recorded; other drugs are then given over wide dosage ranges to assess their abilities to elicit the PCP-like response in the test animals (23). Of many drugs tested in this assay, only a series of PCP analogs and the psychotomimetic opiate SKF 10,047 displayed PCP-like properties; several toxic PCP precursors, including PCC, failed to elicit a PCP-like response even at high doses (H. Shannon, personal communication). It will be of importance to study the involvement of the [^{3}H]PCP-binding site in the anesthetic and analgesic effects of PCP-like drugs; such studies await the ranking of the drugs *in vivo* in these respects.

Together, these findings suggest that some actions of PCP-like drugs *in vivo* may be mediated via binding to a receptor site or other membrane-associated protein. That [^{3}H]PCP

binding is maximal at 4°C and is partially inhibited by sodium make it unlikely that this binding is to an uptake site. The subcellular localization of the binding site suggests that it is associated with synaptic membranes; possibilities include a receptor protein or an enzyme capable of metabolizing PCP. The latter seems unlikely because our thin-layer chromatography study showed that extracted radioactivity after incubation with tissue cochromatographed with pure [^{3}H]PCP.

Other investigators have reported displacement of [^{3}H]QNB and [^{3}H]dihydromorphine from muscarinic and opiate receptor sites by PCP and some of its derivatives (14, 15). In contrast, as we report here for [^{3}H]PCP binding studied directly, alkaloid and peptide opiate-receptor ligands (with the exception of SKF 10,047) were inactive at 0.1 mM. Muscarinic ligands were weak displacers of [^{3}H]PCP, with IC_{50} values about two to three orders of magnitude weaker than the IC_{50} of PCP itself. Moreover, these ligands displaced [^{3}H]PCP in rank order of potency (oxotremorine > arecoline > scopolamine > QNB, atropine) completely at variance with that reported for displacement of [^{3}H]QNB in the muscarinic system (atropine > QNB > scopolamine > oxotremorine > arecoline) (21). Thus, we feel it unlikely that the [^{3}H]PCP binding involves interactions at opiate or muscarinic sites or that such interactions can account for the primary effects of PCP and its analogs. Attempts to detect binding of PCP and related drugs at central nervous system receptor sites for glycine, γ-aminobutyric acid, glutamate, dopamine, serotonin, and norepinephrine have yielded negative results (15).

The binding assay described here should prove useful in screening drugs for PCP-like activity. In addition, such an assay may permit development of specific antagonists that could prove useful in developing pharmacological treatment strategies for psychotic and toxic states induced by PCP and related compounds. Finally, a search for an endogenous ligand for the PCP binding site may be warranted in view of the discovery of such ligands for the opiate (24, 25) and possibly the benzodiazepine (26, 27) receptors. In view of the spectrum of action of PCP and related drugs, such a ligand could play an important role in varied processes of the central nervous system.

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